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SUBTITLE: Evaluation of the Utility of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity and Evaluating Antivesicant Treatment Regimens

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13. ABSTRACT (*Maximum 200 words*)
Two human skin equivalents (HSEs) and dermatomed natural human skin (NHS) were examined as models for investigating the dermatotoxic effects of sulfur mustard (HD). The HSEs examined were from Advanced Tissue Sciences (ATS, La Jolla, CA) and MatTek Corporation (Ashland, MA). NHS was obtained either fresh or cryopreserved from local suppliers. Each skin model was assessed by histopathology and by several viability indices. Tissue samples were examined by both light and electron microscopy for consistency in exhibiting normal ultrastructural details. The viability indices included glucose utilization, lactate production, nicotinamide adenine dinucleotide (NAD^+) content, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction.
Histologically, necrosis was seen in ATS HSE samples. Samples did not exhibit interlot consistency and frequently lacked a continuous basement membrane. The MatTek sample exhibited more interlot consistency, both histologically and by viability indices, but completely lacked a fibroblast layer and basement membrane. Both HSE models were deemed unsuitable for dermatoxicity studies involving HD.
Dermatomed NHS samples, both fresh and cryopreserved, appeared to be fairly consistent from donor to donor. These tissues were judged to be histologically adequate for up to three days after receipt. Cryopreserved NHS appeared histologically normal.

14. SUBJECT TERMS
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Executive Summary

Two human skin culture models, also known as human skin equivalents (HSEs), and dermatomed (split thickness) natural human skin (NHS) were assessed for use in studies investigating the dermatotoxic effects of sulfur mustard [bis(2-chloroethyl) sulfide; HD]. The HSEs examined were model ZK1300 from Advanced Tissue Sciences (ATS, La Jolla, CA) and Epiderm® from MatTek Corporation (Ashland, MA). NHS specimens were obtained either fresh from the Ohio State University branch of the Cooperative Human Tissue Network (Columbus, OH), or cryopreserved from the Ohio Valley Tissue and Skin Center (OVTSC, Cincinnati, OH). The utility of each skin model was assessed by histopathology and by several viability indices. Tissue samples were processed for both light and electron microscopy and examined for consistency among HSE lots and among NHS patients in exhibiting normal ultrastructural details. In particular, the samples were examined for the presence of a continuous, intact basement membrane and an epidermal-dermal interface resembling that found in normal, living human skin. The viability indices included glucose utilization, lactate production, nicotinamide adenine dinucleotide (NAD^+) content, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction. Detailed methods for these assays are included in this report and constitute an important contribution to the assessment of skin viability.

In general, histologic assessments indicated that the ATS HSE was less than satisfactory. Necrosis was prevalent, even on the day of receipt at Battelle's Medical Research and Evaluation Facility (MREF), and the tissues exhibited relatively low interlot consistency in histologic details. This model did not exhibit consistently a continuous, healthy basement membrane needed for studying the effects of HD. The MTT reduction endpoint, commonly used in quality control of skin culture preparations, may not in fact be an accurate index of epidermal health. The histologic data, correlated with the MTT scores, suggested that a healthy fibroblast layer could produce high MTT reduction scores even when the epidermis exhibited extensive cellular degeneration.

The MatTek model was more consistent from lot to lot, both in terms of histologic details and viability indices, but completely lacked a fibroblast layer and basement membrane, features intimately involved in blister formation in humans. Both HSE models were found to be unsuitable for dermatoxicity studies involving HD.

Dermatomed NHS samples, both fresh and cryopreserved, appeared to be fairly consistent from patient to patient, and these tissues were judged to be histologically adequate for studies up to three days after receipt. The most prominent drawback to using fresh NHS was lack of availability in quantities needed to perform experiments with adequate controls. Cryopreserved NHS was available from the OVTSC in sufficient quantities and appeared to be histologically normal.

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TASK 91-24:
EVALUATION OF THE UTILITY OF HUMAN SKIN EQUIVALENTS FOR STUDYING HD-INDUCED DERMATOTOXICITY AND EVALUATING ANTIVESICANT TREATMENT REGIMENS

1.0 Introduction

The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) has considered using cultured human skin tissue models for studying the dermatotoxicity of sulfur mustard (HD). Battelle's Medical Research and Evaluation Facility (MREF) was assigned Task 91-24 to:

- evaluate two commercially available human skin equivalents (HSEs) and natural human skin (NHS) for use in dermatotoxicity studies,
- establish quality control databases for several tissue viability endpoints, and
- use one of the skin models to determine its response to exposures of dilute HD.

The task assigned originally consisted of five phases:

Phase	Subject of Investigations
I	Evaluation of intra- and inter-lot variability for both HSEs and NHS
II	HD vapor dose-response studies for both HSEs and NHS
III	Time course studies at fixed HD vapor doses for both HSEs and NHS
IV	HD liquid concentration-response studies for one HSE
V	Time course studies at fixed HD liquid concentrations for one HSE

Materials and methods employed in this study are presented in MREF Protocol 94 (Appendix A of this report), titled "Evaluation of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity". Appendix B is an interim report dated October 28, 1994 which presented the results of Phase I, i.e., investigations of the following tissues:

- Skin$^{2™}$ model ZK1300 HSE, produced by Advanced Tissue Sciences (ATS, La Jolla, CA), formerly Marrow-Tech. This model consists of a fine nylon mesh first seeded with human neonatal fibroblasts and then covered with keratinocytes, which

differentiated into an epidermis complete with a stratum corneum and basal, spinous, and granular layers;

- Epiderm® HSE, produced by MatTek Corporation (Ashland, MA). This model consisted of human epidermal keratinocytes seeded onto a collagen-modified, microporous Teflon® substrate and allowed to differentiate into an epidermis with a stratum corneum;

- natural human skin (NHS) obtained from The Ohio State University branch of the Cooperative Human Tissue Network (Columbus, OH). Skin sections were dermatomed at the MREF to a target thickness of 1 mm and maintained in culture media at 4 C.

A change in the scope of work for this task required a reassessment of ZK1300 HSE after ATS modified a production step, as well as an evaluation of NHS samples obtained from a different supplier, the Ohio Valley Tissue and Skin Center (OVTSC, Cincinnati, OH). This final report includes a synopsis of the conclusions discussed in the interim letter report (Section 2.0) and a description of the subsequent additional work (Section 3.0).

2.0 Synopsis of Letter Report No. 1

2.1 Materials and Methods

Three tissue types of human skin were obtained and maintained in nutrient solutions at the MREF. Histologic processing and assessments for viability were performed at prescribed time intervals after receipt. Slides of tissues were sent to histopathologists for assessment of viability at each time interval. HSE samples from ATS (four lots) and MatTek (eight lots) were

processed on Day 0 (the day of receipt), and one lot of each HSE type was processed on Days 1, 2, and 3. NHS samples from four patients were processed on Day 0, and the sample from one patient was processed on Days 3, 5, and 7. Portions of the specimens were fixed in formalin, embedded in paraffin, cut into approximately 5 μm thick slices, mounted on glass slides, and stained with hematoxylin and eosin. The slides were sent to Dr. Arthur E. Pellegrini at The Ohio State University Hospitals for examination by light microscopy (LM). Other portions of tissues were fixed in half-strength Karnovsky's fixative and shipped to North Carolina State University (NCSU) for routine plastic embedding. Dr. Nancy A. Monteiro-Riviere at the Cutaneous Pharmacology and Toxicology Center of the College of Veterinary Medicine at NCSU examined these tissues by transmission electron microscopy (EM).

Biochemical viability analyses were performed on four lots of each HSE type on Day 0, but only on one lot of each HSE type on Days 1, 2 and 3. The analyses were performed on two NHS samples on Day 0, and on one NHS sample on Days 3, 5, and 7. The viability analyses included glucose utilization, lactate production, nicotinamide adenine dinucleotide (NAD^+) content, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction. Whereas glucose utilization is a general index of cellular metabolism, lactate is a product of anaerobic metabolism only. NAD^+ is an electron carrier in both glycolysis and oxidative phosphorylation; thus, NAD^+ concentration is used to indicate the metabolic state of a tissue. MTT reduction occurs specifically in the mitochondria and may be used to assess mitochondrial activity.

2.2 Results

2.2.1 Natural Human Skin

Tissue inspection by LM revealed that NHS suffered relatively little deterioration from the day of receipt to 7 days later. No necrosis was observed in the NHS tissue, but some vascular spaces were observed in the upper dermis on Days 5 and 7. EM indicated

intercellular epidermal edema in Day 0 samples, which was not observed on LM. The EM report indicated that the overall integrity of the NHS on Day 0 was good, but cellular degeneration increased with time. Glucose utilization and lactate production levels in samples from one patient decreased over the 7-day period to approximately 75 percent of Day 0 values. The ratio of lactate units produced per glucose unit used remained at approximately 30 percent for 7 days. NAD^+ concentrations decreased dramatically over the 7-day period to approximately 17 percent of Day 0 values. However, MTT scores for that patient's skin remained relatively stable. MTT scores for a different patient's samples decreased with time. Thus, NHS tissue exhibited considerable stability in terms of ultrastucture, glucose utilization, and lactate production (anaerobic metabolism), but showed signs of NAD^+ depletion and cellular degeneration at the EM level. This was the only model of the three examined that was considered sufficiently stable for use in HD dermatotoxicity studies.

2.2.2 Advanced Tissue Sciences Human Skin Equivalent

By LM, the ATS HSE samples within each lot were more variable than NHS specimens from a given patient, but variability across lots was not apparent. Only one lot revealed necrosis, which was minimal in all of the samples. ATS HSE epidermal tissue proliferated with time. The EM report was more critical of the ATS tissue. In general, either the fibroblast layer or the epidermis appeared healthy, with one cell type apparently thriving at the expense of the other. The MTT reduction assay may not be a valid index of overall tissue viability for this reason. MTT scores obtained on HSE lots at the MREF were frequently similar to those obtained by ATS during routine quality control procedures prior to shipment. These MTT scores indicated healthy tissue. Yet, histologic examination revealed that whereas the fibroblast layer appeared healthy, the epidermal layer was inferior. Vacuolization within the stratum corneum and epidermis, spaces in the dermal matrix, and a discontinuous basement

membrane were cited as observations that invalidated this model for studying HD dermatotoxicity. Metabolic indices on Day 0 were similar across lots except for lots suspected of damage from exposure to cold weather during shipment. The glucose, lactate, and NAD^+ endpoints indicated a surge in the metabolism of one lot monitored for three days, which confirmed the epidermal proliferation observed by LM. The EM report identified serious histologic flaws in this model that discourage its use in HD dermatotoxicity studies.

2.2.3 MatTek Human Skin Equivalent

MatTek HSE lots may have been stressed by shipping conditions, as evidenced by dyskeratosis/necrosis in some Day 0 LM samples, although metabolic indices showed no signs of stress in those samples. Other, apparently healthy samples demonstrated increasing keratin layer thickness and decreasing epidermal thickness with time. Tissue inspection by EM indicated that the overall integrity of the Day 0 samples was good, but cellular degeneration increased between Days 1 and 3. This HSE had a poorly developed "pseudo basement membrane" and lacked the anchoring structures needed to assess HD damage at the dermal-epidermal junction. Metabolic indices on Day 0 were uniform across lots. One lot monitored for 3 days exhibited decreases in metabolism endpoints; Day 3 values ranged from approximately 37 to 67 percent of Day 0 values. This correlated with the cellular degeneration noted on EM and slight necrosis and rare to focal vacuolation noted on LM.

In summary, future studies employing any of these models should be performed with the freshest tissue possible, as degradation progressed with storage time in all models. The interlot variability in metabolic indices for MatTek HSE samples was acceptable when shipping conditions were controlled. Both HSE models lacked the ultrastructural features needed for making histopathologic assessments of the effects of HD. NHS up to 3 days old

could be used if sufficient quantities of skin from the same patient could be obtained.

3.0 Latest Studies

The remainder of this final report presents the results from metabolic and LM assessments of a revised ATS ZK1300 HSE model, and LM assessments of NHS obtained from the OVTSC.

3.1 Materials and Methods

3.1.1 Tissue Receipt

Four lots of ATS HSE tissue were received. Each shipment arrived with 24 samples per carton in a styrofoam container with a gel pack to provide thermal stability. The tissue samples were 11-mm squares, each packed in a microplate well with approximately 5 mL of nutrient agarose. Each sample was gently removed from the agarose with forceps and placed on a Millicell insert in a well with 1 mL of prewarmed ATS maintenance media (Catalog No. ZM1060), which was replaced daily.

NHS was obtained from the OVTSC in order to evaluate cryopreserved skin, available in large quantities. All NHS samples were from a 47-year old male who had died from cardiopulmonary arrest. Samples were wrapped in gauze soaked in minimum essential medium containing 5 percent fetal calf serum and either frozen at approximately -70 C or stored for up to 24 hr at 4 C. The frozen samples were shipped on dry ice in a styrofoam container. The unfrozen samples were shipped with a cold pack to maintain the temperature in the styrofoam container at just above 0 C. The frozen samples were thawed while inside the sealed packaging in a 37 C water bath. The period between the date of the donor's death and the date of tissue processing for light microscopy was eight days. Unused portions of NHS samples were rolled up in a sterile gauze pad and placed into a centrifuge tube with 20 mL of ATS maintenance media and stored at 4 C.

3.1.2 Sampling and Processing Tissues for Histologic Examination

Histologic specimens from ATS HSE tissue were collected on Days 0, 1, and 2. On each day, tissues were placed on a polycarbonate cutting board wetted with assay medium and samples taken with a 5-mm diameter biopsy punch (Accuderm Inc., Ft. Lauderdale, FL). ATS also sent two samples of HSE that had been placed into 5 percent neutral buffered formalin solution just before shipping to allow histologic comparison of tissue quality before shipping with that after arrival at Battelle. The split-thickness NHS also was sampled on Days 0 and 1, using a 5-mm skin punch. One sample from each NHS was placed into 5 percent neutral buffered formalin solution. Formalin-fixed specimens were embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. ATS HSE slides were examined by Dr. Monteiro-Riviere, and NHS slides were examined by Dr. Pellegrini.

3.1.3 Viability Assessments

ATS HSE tissues were screened upon receipt by assessing viability. The sample size was three or four replicates per HSE lot for each of the viability assays. Methods for each of these assays are briefly described here. The NHS samples were not screened for viability.

3.1.3.1 Glucose Utilization and Lactate Production Rates

These metabolic endpoints were determined simultaneously with the same instrument, a Model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). Analyses were performed on intact, 11-mm square HSE samples. The initial volume of maintenance medium was 1.0 mL. The medium was changed in the holding well and replaced after 1 hr. This step was necessary to remove any lactate that had accumulated during overnight

incubations. Thereafter, 55-µL aliquots were collected from each specimen well at hourly intervals for 4 hr. The sampled medium was not replaced, but the assay values were corrected for this repeated loss of volume. The samples were analyzed for glucose and lactate concentrations. The values were plotted against time from 1 to 4 hr after the nutrient medium change to estimate slopes representing metabolic rates.

3.1.3.2 MTT Reduction

MTT reduction determinations were performed on intact, 11-mm square HSE samples. The tissue specimen was placed into a plastic well with 2 mL of 2 mg/mL MTT in ATS assay medium, covered, and incubated in a 5 percent carbon dioxide, 90 percent relative humidity atmosphere at 37 C for 2 hr. The solution was then removed, and the specimen was washed twice for 2 min with 1 mL of phosphate buffered saline. The reduced dye was extracted from the cells over a 1-hr period with 4 mL of isopropanol, and a 100-µL sample of the extract was placed into a 96-well plate. A 100-µL volume of isopropanol was added to reduce absorbance, measured at 540 nm, to a range of standards. See Attachment 7 in Appendix B for more details on the MTT assay.

3.1.4 Statistical Analysis

Viability data were organized in a database program that was used to sort records by tissue type, specimen size, and test day. The data were loaded into a spreadsheet program that calculated univariate statistics. Slope estimates for glucose utilization and lactate production were calculated by linear regressions as a function of time using the data from four samples assayed for a given lot. Dr. Pellegrini's LM report on the NHS samples was brief and is presented *in toto* in Appendix C. Dr. Monteiro-Riviere's measurements of strata thickness in ATS samples were

tabulated across replicate specimens for a given lot and sample day. Descriptive statistics were computed for stratum corneum, epidermis, and total skin thickness.

4.0 Results

4.1 Assessment of Natural Human Skin by Light Microscopy

Dr. Pelligrini summarized the differences between Days 0 and 1 as a widening of dermal perivascular spaces and a degeneration of sweat apparatus epithelium. He noted no differences between 8-day old cryopreserved and unfrozen skin specimens, although all slides were clearly identified as one type or the other. There was no mention of either necrosis or tissue variability in his report (Appendix C). Apparently, cryopreserved samples from OVTSC offer a suitable model of healthy skin for studying the dermatotoxic effects of HD.

4.2 Advanced Tissue Sciences Human Skin Equivalent

4.2.1 Assessment by Light Microscopy

A summary of descriptive statistics on the thickness of three tissue layers observed in histologic sections of ATS HSE model ZK1300 is presented in Table D-1 of Appendix D. Raw data for this table are presented in Dr. Monteiro-Riviere's report in Appendix E.

The mean thickness of Day 0 tissue layers ranged from 6 to 11 μm, 41 to 51 μm, and 151 to 179 μm for stratum corneum, epidermis, and total skin, respectively. The interlot variability of these samples, represented in Table D-1 (Appendix D) by coefficient of variation (relative standard deviation), ranged from 0.07 to 0.27 for the three thickness measurements. Dr. Monteiro-Riviere considered this level of variability a deficiency in this model for use in dermatotoxicity studies.

Samples of Lot No. 8043-3-104-11-15D from Days 1 and 2 were examined and

.demonstrated general thinning of the epidermis and of the entire skin section relative to Day 0. The stratum corneum layer was poorly developed on Day 0 and did not improve.

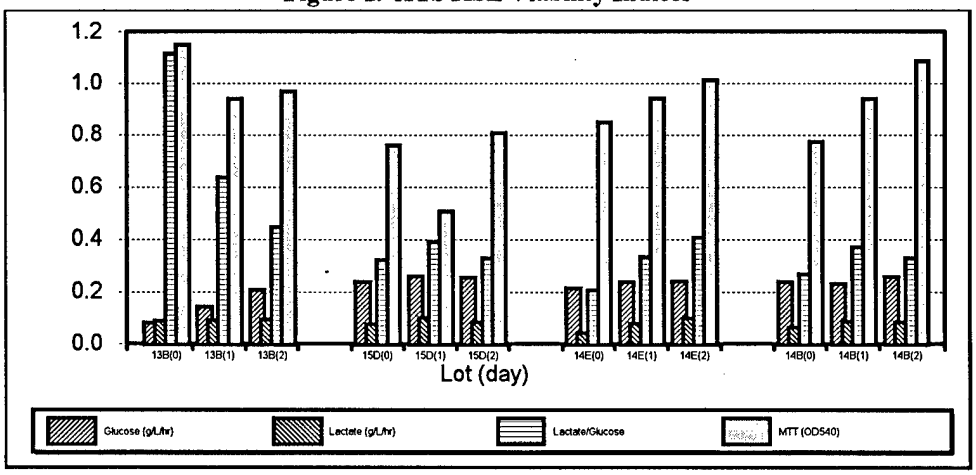
Two samples of Lot No. 8046-3-138-11-14B that were placed into formalin before shipping had a thicker epidermis (53 μm) than four samples prepared on Day 0 (47 μm), but this difference was within the range of intralot variation. Thus, shipping did not seem to have a significant impact on tissue thickness.

Dr. Monteiro-Riviere concluded that the "overall morphology [of these histologic samples was] very poor" and " tissue damage and intercellular and intracellular epidermal edema were often so severe that the samples could not be favorably compared to normal skin." The morphologic condition of the samples varied considerably from lot to lot, and this model was deemed unsuitable for use in dermatotoxicity studies.

4.2.2 Assessment by Viability Indices

A summary of descriptive statistics on rates of glucose utilization, lactate production, and MTT reduction are presented in Table D-2 (Appendix D). The mean results are presented in Figure 1.

Figure 1. ATS HSE Viability Indices



Glucose utilization for HSE samples on Day 0 ranged from approximately 0.08 to 0.24 g/L/hr, and tended to increase (0.21 to 0.26 g/L/hr) over the next two days. Lactate production initially ranged from approximately 0.04 to 0.09 g/L/hr and tended to stabilize by Day 2 between approximately 0.08 and 0.10 g/L/hr. The amount of lactate produced per unit glucose utilized (an index of anaerobic metabolism) ranged on Day 0 from 0.21 to 1.11, but stabilized by Day 2 at approximately 0.33 to 0.45. The complement of this, 0.55 to 0.67, represents the portion of glucose metabolized by aerobic pathways. MTT assay optical density at 540 nm (OD_{540}) means ranged on Day 0 from approximately 0.76 to 1.15 and tended to increase over the next two days to approximately 0.81 to 1.09. All of these indices indicated a slight increase in metabolism and subsequent homeostasis after removal of tissue from the nutrient agarose used during shipping and placement in maintenance media at 37 C. The interlot variability of these viability indices, represented in Table D-2 by the coefficient of variation, tended to decrease with time after tissue delivery. This indicated that the metabolic state of the samples, although dissimilar upon arrival, tended to reach relative conformity after a day or two in maintenance media. Thus, ATS HSE tissue maturation consisted of a general slight increase in metabolic rate with increasing interlot conformity, as well as a general minimal thinning of tissue layers.

5.0 Conclusions

Examination by light microscopy of histologic samples of cryopreserved natural human skin obtained from the Ohio Valley Tissue and Skin Center in Cincinnati, OH indicated that this model may provide the required histomorphology for use in dermatotoxicity research. Although more samples of cryopreserved skin should be examined before drawing a conclusion about this preparation method, the evidence at hand does not preclude its use in future studies. Examination of samples of ATS ZK1300 human skin equivalent indicated this model to be unsuitable for dermatotoxicity studies due to low lot-to-lot consistency, more in terms of normal histomorphology than in viability.

6.0 Record Archives

Records pertaining to the conduct of Task 91-24 are contained in Battelle laboratory record notebooks. All original data will be maintained at Battelle until forwarded to USAMRMC or archived at the conclusion of the task.

7.0 Acknowledgements

The name, role in the study, and highest academic degree of each of the principal contributors in this study are:

John B. Johnson	MREF Manager	D.V.M.
Thomas H. Snider	Study Director*	B.S.
David W. Hobson	Principal Investigator	Ph.D.
Christopher A. Logel	Study Technician	B.S.

*Previous to Mr. Snider, Dr. James A. Blank, Ph.D. served as this project's Study Director.

APPENDIX A

MREF Protocol 94

Evaluation of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity

Evaluation of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity

Study performed by Battelle Columbus Operations,
505 King Avenue, Columbus, OH 43201-2693

1. MREF Principal Investigator and Manager: David W. Hobson, Ph.D., D.A.B.T.

2. Study Director: James A. Blank, Ph.D.

3. Study Supervisor: Thomas H. Snider, B.S., D.A.B.T.

4. Study Dermato-Histologist: Nancy A. Monteiro-Riviere, Ph.D.

5. Study Dermato-Pathologist: Arthur Pellegrini, M.D.

6. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

7. Sponsor Monitor: LTC Don W. Korte, Jr., U.S. Army Medical Research Institute for Chemical Defense (USAMRICD)

8. Background: A primary mission of the USAMRDC program is to characterize the pathophysiology produced by sulfur mustard (HD) in order to design effective therapeutic interventions. HD is a bifunctional alkylating agent which possesses vesicating properties. Although HD, like many other alkylating agents, is thought to exert its toxic effects primarily through irreversible binding to deoxyribonucleic acid (DNA), the mechanism of HD-induced vesication is unknown. A number of biochemical parameters, such as poly(ADP-ribose) polymerase (PADPRP) activation, nicotinamide adenine dinucleotide (NAD^+) depletion, adenosine triphosphate (ATP) depletion, decreased glucose utilization, increased lactate production, and enhanced tissue-associated protease activity, have been associated with HD-induced pathology. Suitable in vitro models for the routine testing and evaluation of treatment compounds have not been identified. Human skin equivalent (HSE) test systems are now available commercially in quantities sufficient for routine testing. Findings from preliminary evaluation of an HSE test system by the USAMRICD indicates that HSEs may be useful models for studying various aspects of HD pathophysiology. A thorough evaluation of the HSE test systems currently available on a commercial basis would further define the in vitro models and allow a decision to be made concerning their utility in testing and evaluating therapeutic regimens for HD-induced dermatotoxicity.

9. Objective: The objective of studies conducted under this protocol for MREF Task 91-24, "Evaluation of the Utility of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity and Evaluating Antivesicant Treatment Regimens", is to evaluate commercially available HSEs as an in vitro system for evaluating therapeutic regimens and for studying HD pathophysiology.

10. Experimental Overview: The responsiveness of the HSEs to HD-induced histological and biochemical alterations are examined and compared to the responsiveness of natural human skin, assuming a sufficient quantity can be obtained. Histological changes are assessed by light microscopy (LM) performed by Dr. Arthur Pellegrini and by transmission electron microscopy (TEM) performed by Dr. Nancy Monteiro-Riviere. As shown below, Task 91-24 can be divided into five phases with a decision point occurring between phases. Each phase is initiated as directed by the Contracting Officer's Representative (COR).

PHASE I
Evaluation of Intra- & Inter-lot
Variability for HSEs and
Natural Human Skin

↓ ⟶ Letter Report and
Decision Point #1

PHASE II
HD Vapor Dose Response Studies for
HSEs and Natural Human Skin
(Determine Fixed HD Doses)

↓ ⟶ Letter Report and
Decision Point #2

PHASE III
Time Course Studies at Fixed HD Vapor
Doses for HSEs and Natural Human Skin

↓ ⟶ Letter Report and
Decision Point #3

PHASE IV
HD Liquid Concentration
Response Studies for One Source of HSE

↓ ⟶ Letter Report and
Decision Point #4

PHASE V
Time Course Studies at Fixed HD
Liquid Concentrations for One Source of HSE

11. Experimental System: At present, Advanced Tissue Sciences (ATS; La Jolla, CA) is the only commercial source of HSE. Once a second source of HSE is identified, it may also be evaluated as directed by the COR.

    A. Natural Human Skin - Natural human skin is obtained through the Cooperative Human Tissue Network (CHTN), which is locally operated through The Ohio State University. The tissue can be purchased through the CHTN and there are no unique patient identifiers associated with the appropriated tissue. Upon receipt, skin specimens are dermatomed to a thickness of 1 mm. The specimens may then be stored for the length of time determined acceptable in Phase I. These human skin specimens are handled in accordance with Battelle SOP H/SP IV-003 pertaining to biohazardous materials.

    B. ATS HSE - ATS Model ZK-1300 Kit is used for these studies. This is a full-thickness skin equivalent system which contains a dermal layer comprised of fibroblasts and a stratified epidermal layer comprised of keratinocytes grown on a nylon mesh. The human fibroblasts and keratinocytes used in the production of this HSE are pooled across 10 donors. Cells pooled from these donors may be used in the production of many HSE kits. As such, a lot for a given kit is based upon the kit manufacturing date (i.e., when the HSE culture was initiated).

        (1) Each kit contains 24, 11 by 11 mm HSE sections.

        (2) This material is handled aseptically to the maximum extent possible. Upon receipt, the material is unpackaged and the lot number of HSE and media provided is documented. Maintenance Medium (ATS) provided in the kit is warmed in a 37 C, 5 percent carbon dioxide incubator for a minimum of 30 min. One mL of Maintenance Medium is placed per well of the six well plates provided. The HSE section is gently grasped at an edge with a pair of fine-tipped forceps, rinsed in Maintenance Medium, then placed on the filter support lying at the air-liquid interface.

        (3) The HSE may be maintained in culture for up to three days, with spent medium being removed and replaced with fresh, pre-warmed Maintenance Medium each day. The effect of this storage or culturing process on tissue quality is examined under Phase I experimental studies.

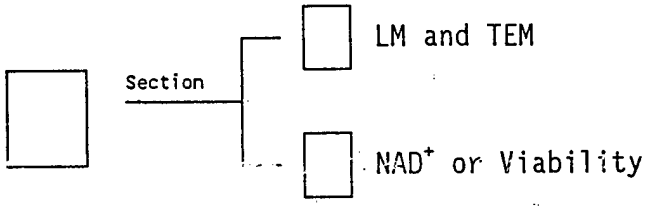
12. Experimental Design:

    A. Phase I: Intra- and Inter-Lot Variability Studies: Studies are performed to assess the intra-lot and inter-lot histological variation of natural human skin and HSE from up to two sources. As these

tissues are to be received and used on different days, studies to evaluate the impact of storage on tissue histology are also performed. Measured parameters involve LM and TEM assessments, but may also include tissue NAD^+ content, tissue viability, glucose utilization, and/or lactate production to enhance system characterization. HSE from one commercial source is examined initially, followed by a second HSE type assuming commercial availability. Natural human skin is examined as the tissue becomes available. As described below, the intra- and inter-lot differences of tissue are examined on Day 0 (i.e., when the tissue is received) and up to three time periods of storage are examined.

(1) LM and TEM examinations are performed on three HSE samples taken from each of four different HSE lots when the tissue is received on Day 0. For natural human skin, three samples of tissue from each of four donors are taken upon receipt of the tissue for examination. Due to the availability of different HSE lots and natural human skin, samples to evaluate inter-lot and inter-individual differences are collected on different days.

a. ATS HSE is sectioned as shown below and processed as described in Section 13 for histological evaluation.



b. Twelve specimens (three samples from each of four lots) from each of two HSE types and natural human skin (three samples from each of four donors) are examined for a total of 36 specimens for Day 0 evaluations.

(2) To assess the impact of storing the HSEs prior to use, three samples from each HSE lot and one donor skin that have been maintained in culture, will be taken on each of three consecutive days and examined microscopically. This yields an additional 9 specimens for each HSE and natural human skin or a total of 27 specimens for examining the impact of storage on tissue quality.

(3) HSE sections and natural human skin sections are placed in the proper fixative solutions and submitted for evaluation as described in Section 13. Phase I generates 21 specimens per HSE and natural human skin for both LM and TEM evaluation for an all-inclusive total of 63 specimens for Phase I studies.

NUMBER OF SAMPLES COLLECTED FROM A SPECIFIC LOT (HSE) OR DONOR FOR LIGHT AND TRANSMISSION ELECTRON MICROSCOPIES IN PHASE I STUDIES

	Day			
Skin Type	0	1	2	3
HSE, Source 1	3, 3, 3, 3*	3	3	3
HSE, Source 2	3, 3, 3, 3	3	3	3
Donor 1	3	3	3	3
Donor 2	3			
Donor 3	3			
Donor 4	3			

* Three samples from each of four lots.

(4) Following receipt of LM and TEM results, a letter report describing the findings of this phase is submitted to USAMRICD. A decision is made in conjunction with the USAMRICD technical Point of Contact (POC), Task Area Manager (TAM), and COR as to whether the natural skin and HSE quality warrants use in further studies.

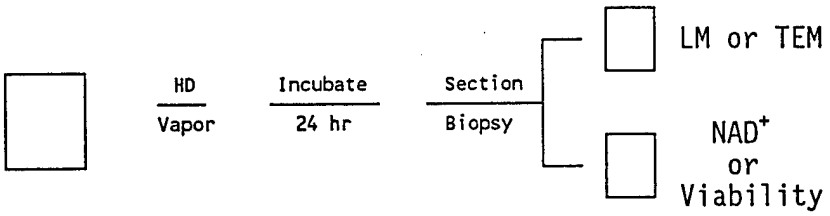
B. Phase II: HD Vapor Exposure Time-Response Studies: The purpose of this experimental phase is to establish the relationship between HD vapor exposure times and NAD^+ depletion and histological alterations at 24 hr after exposure for natural human skin and two HSE types. The objective of this phase is to determine a fixed vapor exposure time(s) for use in the conduct of Phase III studies.

HD vapor concentrations are estimated analytically, following the procedures of Battelle SOP MREF III-002, before these studies are initiated. These studies entail examining the proper sampling technique for an occluded environment. Once established, the length of time required for maximal HD vapor generation and the maintenance of this vapor concentration when the cap is in place over an HSE are examined. Once the exposure conditions are established, then the studies to establish the vapor exposure time-NAD^+ and histological responses are conducted.

(1) HD exposures are performed by vapor cup as described in Section 13.

(2) A stage-wise experimental approach is used to perform the vapor exposure time-response study. A minimum of three stages is used to establish the relationship, with the initial stage consisting of vapor exposure times ranging from 0.5 min to 14 min. The data from the initial stage are evaluated, then the exposure times for subsequent stages are selected.

(3) The tissues are exposed, incubated at 37 C (± 5 C), 5 percent carbon dioxide (± 0.5 percent), saturated humidity atmosphere for 24 hr, then examined for NAD^+ depletion, tissue viability, and histological alterations. A section of the tissue is placed in fixative for LM and TEM evaluations, and other sections are assayed for NAD^+ content or viability as described in Section 13. The tissue is sectioned as described in Section 13. Samples for histopathological evaluation are clearly marked with project number, protocol number, date, fixative, material, and sample identification. A sample identification sheet (Attachment A) is also completed, a copy is submitted with the samples, and the original is retained for study files.



(4) These studies may yield up to 72 specimens for LM evaluation. TEM evaluations are performed on a limited number of samples. For each HSE or natural human skin, three specimens at each of four different HD exposures times (including a non-exposed group) may be submitted for TEM evaluation (e.g., control and three HD exposure times which cause varying degrees of NAD^+ depletion). This yields 12 TEM evaluations for each HSE or natural human skin for a total of 36 TEM evaluations for Phase II studies.

(5) The HD vapor exposure times required to reduce NAD^+ by 25 percent (VT_{25}), 50 percent (VT_{50}) and 75 percent (VT_{75}), as well as a no observable effect level (NOEL) at 24 hr post exposure are estimated as described in Section 13.

C. Phase III - Time-Course Studies Following HD Vapor Exposures: Time-course studies measuring NAD^+ and ATP levels, PADPRP activity, glucose utilization, lactate production, tissue-associated protease activation using two chromogenic substrates (chromozymes TH and TRY), and prostaglandin E_2 ($PG\text{-}E_2$), leukotriene B_4 ($Lt\text{-}B_4$), and interleukin-1 (IL-1) release are made for up to two HSE types at four HD vapor

exposure lengths. For the natural human skin, evaluations are performed following HSE evaluations and are limited to two HD vapor exposure levels. The biochemical end points which are found to be the most sensitive to HD in the HSE system are used for the natural human skin.

(1) Unless otherwise directed by the COR, HSE samples are either not exposed, or exposed to the HD VT_{25}, VT_{50}, VT_{75}, and NOEL. Likewise, natural human skin is either not exposed, or exposed to the HD VT_{25} and VT_{75} following procedures described in Section 13. Tissues are exposed to vaporous HD, incubated for varying lengths of time, then sectioned as described in Section 13 for end point measurement. As described in Section 12.C.4, certain assays are also performed on the supernatant from these cultures.

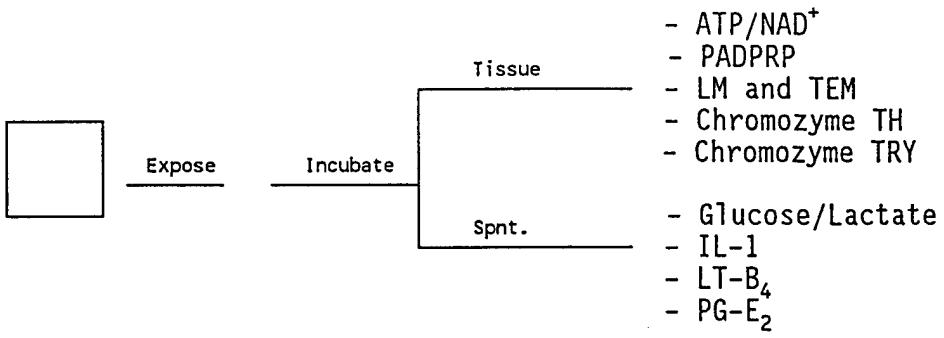
(2) Possible end point alteration is examined at seven time points (e.g., at 0, 2, 4, 8, 12, 16, and 24 hr after exposure to HD vapor) for the HSEs and at four time points for the natural human skin. Due to the limited availability of natural human skin, studies involving the HSEs will be performed first so that the time-course data from the HSEs may be used as an aid in selecting four time points for studies involving the natural human skin. Times will be selected to maximize the range of biochemical effects observed as efficiently as possible.

(3) Each timed-treatment group for the HSEs consists of six replicates. On each day of experimentation, a sufficient number of HSE sections are dosed at each HD exposure level to provide six replicates for each of two or three time points. Since the samples are consumed by the assays, there will be between 48 and 72 exposures per day of experimentation. Exposures for all HD exposure levels are performed as follows:

NUMBER OF REPLICATES AT EACH TIME POINT

	Exposure Day	Time Point		
	1	1	4	7
	2		2	5
Dose Level	3		3	6
1		6	6	6
2		6	6	6
3		6	6	6
4		6	6	6

(4) End point measurements are made on the tissue culture supernatant (Spnt.), and tissue sections (HSE or natural human skin). Although tissue from one exposure is sectioned and used to assess multiple end points, all the listed parameters cannot be evaluated from the same 11 mm by 11 mm piece of tissue. As a result, two to three replicates of the three exposure days (as shown above) for each HSE are required to examine all end points.



(5) Histopathologic changes are examined by LM as well as TEM evaluation on a selected number of samples.

a. LM evaluations are performed on three samples per timed treatment group. For each HSE, there are five HD exposure levels, seven incubation time points, and three observations per timed-treatment group. This yields 105 specimens per HSE or 210 specimens for both HSEs. Natural human skin is examined at three HD exposure levels, four incubation time points, and three observations per timed-treatment group. This yields up to 36 specimens for LM evaluation. In total for Phase III, there may be up to 246 specimens evaluated by LM.

b. TEM evaluations are performed on a limited number of samples. The specimens are prepared and submitted for TEM evaluation as described in Section 13. For each HSE, TEM evaluations are performed on three samples taken at three incubation time periods for a non-exposed and one HD exposed treatment group. This yields 18 specimens per HSE or 54 specimens for both HSEs and the natural human skin under Phase III studies.

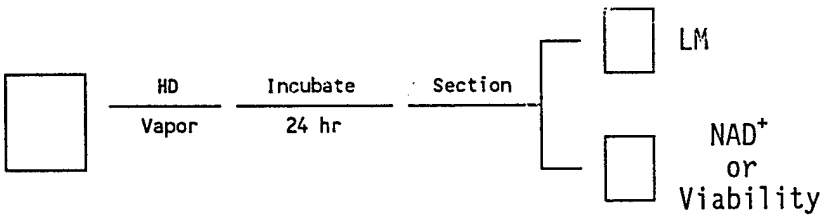
(6) Studies with natural human skin are performed using the end points and time points which appear to be the most sensitive in the HSE systems to HD-induced alteration. Selection of the end points, time points, and the HD exposure lengths for studies involving natural human skin are made in conjunction with the USAMRICD POC, TAM, and COR.

D. Phase IV: HD Liquid Concentration-Response Studies - The purpose of this experimental phase is to establish the relationship between HD concentration, NAD^+ depletion, and histological alterations at 24 hr after exposure. These studies are performed for one HSE type and establish the HD concentrations for use in the conduct of Phase V studies. The HSE type evaluated is selected in conjunction with the COR, TAM, and POC following review of Phase III data.

(1) HD liquid exposures are performed as described in Section 13.

(2) A stage-wise experimental approach is used to perform the HD concentration-response study. In this design, the data from the initial stage are used to select the HD concentrations for subsequent stages. Three stages may be used to establish the relationship.

(3) The tissues are exposed following procedures outlined in Section 13, incubated at 37 C (± 5 C), 5 percent carbon dioxide (± 0.5 percent) for 24 hr, then examined for NAD^+ depletion, viability, and altered tissue histology. Natural human skin and HSE are sectioned and assayed for NAD^+ content or viability as described in Section 13. The sample container is clearly marked with project number, protocol number, date, fixative, material, and sample identification. A sample identification sheet (Attachment A) is also completed, a copy is submitted with the samples, and the original is retained for study files.



(4) These studies may yield up to 48 specimens for LM evaluation. TEM evaluations are not performed on these samples.

(5) The HD concentrations required to reduce NAD^+ by 25 percent (IC_{25}), 50 percent (IC_{50}) and 75 percent (IC_{75}), as well as a NOEL at 24 hr post exposure are estimated using the procedures described in Section 13.

E. Phase V: Time-Course Studies Following HD Liquid Exposures - In this phase, HD liquid exposures are performed examining the end points found to be most sensitive to HD vapor exposures. One HSE type is used, and up to 11 end points may be assessed. The end points are selected in conjunction with the USAMRICD POC, TAM, and COR.

(1) HD liquid exposures are performed as described in Section 13.

(2) The HD concentrations used in these studies are the HD IC_{25}, IC_{50}, IC_{75}, and NOEL for NAD^+ depletion at 24 hr post exposure.

(3) LM evaluations are performed on three samples per timed treatment group. There are five HD exposure levels, seven incubation time points, and three observations per timed-treatment group for one tissue type to yield 105 specimens.

(4) Each timed treatment group consists of six observations. The end points used for these studies are selected in conjunction with the USAMRICD POC, TAM, and COR.

13. Experimental Procedures:

A. HD Exposures

(1) Vaporous - Operations involving HD are performed in accordance with Battelle SOP MREF II-003. HD is dosed onto filter paper inserted inside a cap of sufficient size to cover the majority of the HSE and the cap is affixed onto the HSE for various exposure lengths. The conditions under which the vapor exposures occur are determined mainly from preliminary work conducted as part of Phase II to verify HD vapor concentrations analytically. However, the dermal side of the natural skin or the HSE must be kept moistened. For instance, the natural skin or HSE must be kept on filtered paper contained within a petri plate containing culture medium.

(2) Liquid - Battelle SOPs MREF I-003 and V-003 are followed in this section. If HD concentrations above exempt surety levels are required, Battelle SOPs MREF I-002 and II-003 are followed. A constant volume of HD which has been diluted in tissue culture medium is pipetted onto filter paper of sufficient size to cover the entire HSE or natural human skin section. The filter paper is then placed on the surface of the HSE or natural human skin section with the dosed side of the filter paper coming in contact with the HSE or natural human skin section. At the end of the exposure period, the dosing pad is placed in 5 percent sodium hypochlorite (NaOCl) solution. Alternatively, the HD may be applied as a droplet onto the center of the HSE or natural human skin as directed by the COR.

B. Culturing of HD-Exposed Natural Skin and HSE - Following exposure, the vapor cup is removed from the natural skin or HSE and left in the hood for 1 hr to allow unbound HD vaporization. During this period, the natural skin or HSE again is maintained within the fume hood in an

apparatus such as a petri plate containing filter paper and culture medium.

To demonstrate the level of hazard associated with handling these skin samples, initial samples of HD-exposed, offgassed tissue are placed into scintillation vials each containing 2 mL of hexane with an internal standard. Each vial is vigorously shaken, then opened and aliquoted into three, 1-mL GC vials for chromatographic analysis of HD (Battelle SOP MREF III-002). The individual performs this operation after donning a clean pair of butyl gloves.

Within the fume hood, the material is then transferred to a six-well culture plate apparatus (6-well plate containing 1-mL of assay medium, with Millicell inserts to hold the material at an air-liquid interface). At the clean end of the hood, an individual with a clean pair of butyl gloves places the plates inside a culture box (a petri plate with distilled water is placed on the bottom), the lid is secured, and the box is then gassed with a 95 percent air, 5 percent carbon dioxide mixture for a 2-min period. The air vents are secured and the culture box is placed into a plastic bag held at the hood face by individuals wearing two pair of nitrile gloves. The plastic bag is sealed and then transferred to an incubator. When sample plates are to be removed from the culture box, the box is transferred to a biological safety cabinet or fume hood, the box is opened, and the samples removed. If samples in the box require an additional culture period, the culture box is gassed, the air vents secured, and the box returned to the incubator as described above.

C. Natural Human Skin and HSE Sectioning - Sectioning of skin prior to end point measurement is performed in a fume hood or biological safety cabinet using a straight edge razor for histopathological preparation or with a biopsy punch (e.g., 4 mm diameter) for end point measurement such as NAD^+/ATP, viability, chromozyme substrate metabolism, and PADPRP activity. The individual performing this operation dons two pair of nitrile gloves.

D. Specimen Preparation for Histopathology

(1) Light Microscopy - The fixative for the tissue will be either standard B-5 fixative or Karnoy and Bouin's fixative. After sectioning, an individual donning two pair of nitrile gloves places the material in one of these fixatives for a 2-hr period, then transfers the material to 5 percent neutral buffered formalin and retains the specimens in a fume hood or biological safety cabinet for a 24-hr period. The tissue is transferred to Battelle's histology laboratory (King Avenue) where the tissue is mounted in methyl methacrylate, 5 μm sections are cut, and the slide is stained with Masson-Trichrome stain.

(2) TEM - The minimum specimen size required for TEM is 1 mm wide by 2 mm long section. Within a fume hood or biological safety cabinet, an individual donning two pair of nitrile gloves sections the tissue using a straight-edge razor. The specimen is not to be handled with forceps, but rather it should be handled with an applicator stick. The specimen is placed into a 5-mL vial filled with cold, half-strength Karnovsky's fixative and is placed into a plastic bag which is then sealed and placed in a refrigerator for storage. Half-strength Karnovsky's fixative is an aqueous, buffered solution consisting of 2 percent paraformaldehyde, 2.5 percent glutaraldehyde, and 0.1 M sodium cacodylate, which has a 1-week expiration date from the time of preparation, and is stored refrigerated. After overnight storage (at least 12 hr), the specimens are shipped to North Carolina State University-Battelle Cutaneous Pharmacology and Toxicology Center on wet ice by next day air. Specimens may be held for up to a 3-day period prior to shipping and kept refrigerated until the time of shipping. TEM specimens are sealed in screw-capped vials, surrounded by enough absorbent material to absorb the entire volume of fixative in the shipment, and sealed in a secondary, shock-absorbent container. The secondary container with a warning label in accord with Ohio Department of Transportation regulations, is placed into a cardboard box.

Since Karnovsky's fixative is an inorganic arsenical, unused portions are considered highly hazardous waste and are isolated, tagged, and identified. The waste containers are set aside for special pick up by the Battelle Environmental Safety and Health Department in accordance with Battelle SOP H/SP II-005.

E. End-Point Measurements: Removal of samples from culture boxes are performed in a fume hood or biological safety cabinet by individuals donning two pair of nitrile gloves (Battelle SOP MREF I-003). Tissue samples and supernatants are processed and transferred to clean containers within a clean fume hood or biological safety cabinet. All remaining material is decontaminated in 5 percent NaOCl (Battelle SOP MREF I-003). All sample manipulation for end point measurement purposes is performed within a fume hood or biological safety cabinet, as indicated, by individuals donning two pair of nitrile gloves.

(1) Viability - Viability is assessed by measuring MTT dye uptake and hydrolysis (MREF Method No. 10/In Vitro). The tissue is washed in a physiological salt solution such as phosphate buffered saline. The tissue is placed into MTT solution and transferred to a 37 C (± 0.5), 5 percent carbon dioxide (± 0.5) incubator for a 2-hr period with constant mixing. Within a fume hood or biological safety cabinet, the MTT solution is aspirated and the tissue is washed in a physiological salt solution. The washed

tissue is then digested with solvent and the colored formazan product is measured spectrophotometrically at 540 nm.

(2) Metabolism - Cellular metabolism is assessed by glucose depletion from the medium and lactic acid production. Glucose and lactic acid measurements are assayed both in vehicle control samples which do not contain tissue (background levels) and in the samples containing tissue. Tissue culture supernatant samples are removed from the hood and measurements are made using a Yellow Springs Instrument Biochemical Analyzer by individuals donning two pair of nitrile gloves.

(3) NAD^+ - NAD^+ will be measured following methodology contained in MREF Method No. 1/In Vitro. The method will be modified so as to measure ATP from the same cellular digest. Tissue extractions are performed in a fume hood or biological safety cabinet by individuals donning two pair of nitrile gloves.

(4) ATP - ATP measurements are made on the NAD^+ cellular extracts using a luciferase/luciferin bioluminescent assay. Tissue extractions are performed in a fume hood or biological safety cabinet by individuals donning two pair of nitrile gloves.

(5) $PG\text{-}E_2$, $Lt\text{-}B_4$, and IL-1 - The level of these inflammatory mediators in tissue culture medium are measured using commercially available enzyme immunoassay kits. The tissue culture supernatant is harvested and placed into Eppendorf centrifuge tubes in a fume hood or biological safety cabinet by individuals donning two pair of nitrile gloves. The tubes are centrifuged at 12,000 rpm for 5 min before analysis in a biological safety cabinet by individuals donning two pair of nitrile gloves.

(6) Protease Activity - Tissue associated protease activity is measured using the chromogenic substrates, Chromozym TH and TRY, which have been used in the determination of serine protease activity, particularly thrombin and trypsin, respectively (Cowan et al., 1991) within a fume hood or biological safety cabinet, a section of tissue is placed into assay medium by an individual donning two pair of nitrile gloves. After an incubation period, the tissue is removed and the plate removed from the hood or cabinet and the amount of product is measured spectrophotometrically. The parameters for this assay (i.e., concentration of chromogenic substrate and length of incubation) are to be defined within this task.

(7) PADPRP Activity - PADPRP can modify proteins by attaching the ADP-ribose moiety of NAD^+ to the protein. Within a fume hood or biological safety cabinet, a section of tissue is removed and placed into extraction/solubilization media by an individual donning two pair of nitrile gloves. PADPRP activity is measured following methodology developed during the task by Battelle and USAMRICD investigators. Current USAMRICD procedures use radio-labeled NAD^+ and measure incorporation of label into proteins.

14. Statistical and Quality Control Procedures:

A. HD Dose and Concentration Response Studies - Statistical models are fitted to the HD dose or concentration response data for NAD^+ depletion to estimate the HD VT_{25}s, VT_{50}s, and VT_{75}s, and the HD IC_{25}s, IC_{50}s, and IC_{75}s. Models considered may include standard parametric concentration-response curves with corrections for either natural background levels or limiting responses less than 100 percent. If parametric models are not appropriate for fitting the data then piece-wise linear regression models may be used to estimate the VT_{25}s, VT_{50}s, and VT_{75}s, and the IC_{25}s, IC_{50}s, and IC_{75}s for HD. The NOEL is defined as the highest exposure time or HD concentration evaluated which does not produce a significant ($p \leq 0.05$) amount of NAD^+ depletion at 24 hr post exposure.

B. Time Course/Response Studies - Average levels of HD-induced parameter alteration are plotted against time for HD vapor exposure length or liquid exposure concentration. For each response, separate analyses of variance models may be used to assess the effects of HD vapor exposure level or concentration at each of the sampling time points. If appropriate, analyses of variance with repeated measures on time may be carried out to assess the effects of HD concentration over the duration of the experiment.

C. Quality Control - Assay performance is assessed from tissue sections which are either not exposed or exposed to a level of HD which is known to produce a given histopathological alteration (LM assessment) or a given level of tissue NAD^+ depletion 24 hr following exposure. Both histological evidence of HD exposure and comparison of the NAD^+ response relative to initial experiments are used to assess assay performance. Use of LM evaluations as controls increases the number of LM specimens generated in each of the five phases. If the mean value of the positive assay control (HD-exposed) in the NAD^+ analysis falls outside three standard deviations (STDs) of the mean historical value, then the data are considered suspect. If the cause of the extreme value cannot be determined, then the influence of this experiment on the final results will be assessed before including or omitting the dataset.

15. Record Maintenance: The following records are to be maintained for MREF Task 91-24:

    A. CSM accountability log and inventory,

    B. Reagent preparation,

    C. Decontamination and disposal records, and

    D. Any other records needed to reconstruct the study and demonstrate adherence to this protocol.

16. Reports:

    A. Interim letter reports will be submitted within 30 days following the completion of analysis for each study phase.

    B. At the end of Task 91-24, a Draft Final Report will be prepared and submitted to USAMRICD within 60 working days of task completion. The Draft Final report includes, at a minimum, the following sections:

        (1) Signature page of key study personnel and their responsibilities,

        (2) Experimental design,

        (3) Test material description

        (4) Tabulation and statistical data analysis, and

        (5) Discussion and conclusion.

        The Final Report will be submitted within 30 days of receiving the Draft Final Report comments from USAMRICD.

17. References:

Cowan, F.M., C.A. Broomfield, and W.J. Smith. Effect of Sulfur Mustard Exposure on Protease Activity in Human Peripheral Blood Lymphocytes. Cell Biol. and Toxicol. 7:239-248. (1991).

18. Approval Signatures:

James A. Blank, Ph.D.
Study Director

June 09, 1993
Date

Nancy A. Monteiro-Riviere, Ph.D.
Study Dermato-Histologist
North Carolina State University-Battelle
Dermal Pharmacology Research Center

June 14, 1993
Date

Arthur Pellegrini, M.D.
Study Dermato-Pathologist
The Ohio State University

June 22, 1993
Date

David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager

June 15, 1993
Date

David Stitcher, CIH
Safety and Surety Officer

June 16, 1993
Date

LTC Don W. Korte, Jr.
USAMRICD COR

22 JUN 93
Date

ATTACHMENT A

MREF TASK NO. 91-24
MREF PROTOCOL 94 - HISTOPATHOLOGY SAMPLE RECORD

DATE*	SAMPLE #	PHASE #	SAMPLE DESCRIPTION

* Date of Specimen Collection

APPENDIX B

Task 91-24 Letter Report No. 1

Report Text

Attachment 1: Tables

Attachment 2: Figures

Attachment 3: MREF Method No. 28/*In Vitro*

Attachment 4: MREF Method No. 29/*In Vitro*

Attachment 5: MREF Method No. 30/*In Vitro*

Attachment 6: MREF Method No. 25/*In Vitro*

Attachment 7: MREF Method No. 26/*In Vitro*

Attachment 8: MREF Method No. 27/*In Vitro*

Attachment 9: Dr. Pellegrini's Light Microscopy Report on Advanced Tissue Science Human Skin Equivalent and Natural Human Skin Tissues

Attachment 10: Dr. Pellegrini's Light Microscopy Report on the First Set of Four Lots of MatTek Human Skin Equivalent Tissue

Attachment 11: Dr. Pellegrini's Light Microscopy Report on the Second Set of Four Lots of MatTek Human Skin Equivalent Tissue

Attachment 12: Dr. Monteiro-Riviere's Report with Photographs Characterizing All Three Tissue Types by Transmission Electron Microscopy

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October 28, 1994

LTC Richard R. Stotts, VC, COR
Battelle Columbus Operations
505 King Avenue, Building JM-3
Columbus, OH 43201-2693

Dear LTC Stotts:

Contract No. DAMD17-89-C-9050
Letter Report No. 1 on Task 91-24

Introduction

This letter report presents data from Phase 1 of the Medical Research and Evaluation Facility (MREF) Task 91-24, entitled "Evaluation of the Utility of Human Skin Equivalents for Studying HD-Induced Dermatotoxicity and Evaluating Antivesicant Treatment Regimens". In this phase, tissue quality was examined on natural human skin (NHS) and on two human skin equivalent (HSE) models. The primary objective of this work was to assess tissue quality and variability, both within a lot (or patient) and between lots (or patients), and to assess tissue quality when maintained in a nutrient medium for three days. The HSEs specified in the proposal to be examined were three-dimensional full-thickness models produced by Organogenesis Inc. and Advanced Tissue Sciences (ATS). Following submittal of the proposal for this task, Organogenesis removed their product from the commercial market. Prior to project initiation, an epidermal equivalent HSE produced by MatTek Corporation became commercially available and was included in the task as directed by the Contracting Officer's Representative (COR). An additional, secondary objective of this work was to initiate a database of biochemical measurements of viability for controlling assays in subsequent phases, and to correlate any histologic abnormalities with the viability endpoints.
The endpoints used to evaluate tissue quality were:

- histologic structure at the light microscopy (LM) level performed by Dr. Arthur E. Pellegrini, M.D. at The Ohio State University,

- ultrastructure at the electron microscopy (EM) level, performed by Dr. Nancy A. Monteiro-Riviere, Ph.D. at North Carolina State University (NCSU), and

- viability endpoints performed at the MREF, i.e., glucose utilization, lactate production; nicotinamide adenine dinucleotide (NAD^+) content; and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction.

Materials and Methods

ATS HSE: Model ZK1300 HSE, maintenance medium, and assay medium were purchased from ATS (La Jolla, CA). This ATS HSE model consists of a fine nylon mesh seeded with human neonatal fibroblasts and then covered with keratinocytes, which differentiate into an epidermis complete with a stratum corneum and basal, spinous, and granular layers. This material is maintained at an air-medium interface.

ATS HSE tissue shipments arrived as 24 samples per carton, usually one carton per shipment, in a styrofoam container with a gel pack which served as a heat sink. In early shipments, ATS personnel sent each package from California via Federal Express on Fridays, and Battelle received them the following Monday. During the hot summer months of 1993, this procedure appeared to injure the tissue, presumably due to storage over the weekend in a thermally unregulated warehouse. The stress may have resulted from overheating, or rapid depletion of nutrients and subsequent starvation of the cells. The manufacturer was notified of this problem and began shipping to Battelle on either Saturdays for Monday delivery or Tuesdays for overnight delivery. Regardless of shipment mode, similar problems were encountered. Federal Express does not offer a special package handling service that guarantees thermoregulation.

ATS HSE tissue samples were 11-mm squares, each packed in a microplate well with approximately 5 mL of nutrient agarose. Each sample was gently removed from the agarose with forceps and placed on a Millicell insert in a well with 1 mL of prewarmed maintenance medium (ATS), which was replaced daily. ATS HSE was cultured at approximately 37 C in a 5 percent carbon dioxide, water-saturated atmosphere.

MatTek HSE: Epiderm® Skin Model, Maintenance Medium, and assay medium were obtained from MatTek Corporation (Ashland, MA). This model consists of human epidermal keratinocytes seeded onto a collagen-modified, microporous teflon substrate and allowed to differentiate into an epidermis with a stratum corneum.

MatTek HSE shipments also arrived as 24 samples per carton, usually one carton per shipment, in a styrofoam container with cooled gel pack. MatTek product was packaged and shipped from Massachusetts on Mondays for overnight deliveries on Tuesdays at Battelle. The tissue samples were 9-mm diameter disks, each packed in a Millicell well that was slightly embedded into the surface of approximately 5 mL of nutrient agarose. Each Millicell well with tissue sample was removed from the agarose with forceps, rinsed in MatTek maintenance medium, and placed on the surface of a 1-mL volume of prewarmed maintenance medium which was replaced daily. MatTek HSE was cultured at approximately 37 C in a 5 percent carbon dioxide, water-saturated atmosphere.

NHS: NHS was obtained through the mid-west regional office of the Cooperative Human Tissue Network, an organization supported by the National Cancer Institute. All NHS samples were normal, from female patients with either macromastia or breast sarcoma, ages

29 to 53. Samples were wrapped in gauze soaked in minimum essential medium containing 5 percent fetal calf serum and stored for up to 24 hr at approximately 4 C. Each sample was unpacked, placed on a nutrient-wetted cutting board, trimmed free of subcutaneous fat, and microtomed at approximately 1 mm thickness. Unused portions of NHS samples were rolled up in a sterile gauze pad and placed into a centrifuge tube with 20 mL of ATS maintenance media (Catalog No. ZM1060) and stored at approximately 4 C.

Sampling Tissues for Histologic Examination: Histologic specimens from each HSE tissue type were collected on Days 0, 1, 2, and 3. On each day, three samples were placed on a polycarbonate cutting board wetted with assay medium and perforated twice each with a 5-mm diameter biopsy punch (Accuderm Inc., Ft. Lauderdale, FL). The split-thickness NHS was sampled on Days 0, 3, 5, and 7, and was also biopsied with a 5-mm skin punch. One of the specimens from each sample was placed into B5 fixative solution for 2 hr and then transferred into 5 percent neutral buffered formalin. Formalin-fixed specimens were processed, embedded in paraffin, sectioned at 5 μm thickness, stained with hematoxylin and eosin (H&E), then taken to Dr. Pellegrini for routine histologic evaluation. The other specimen from each sample was split with a razor, placed into half-strength Karnovsky's fixative at 4 C, then shipped to NCSU on wet ice for routine plastic embedding and transmission EM evaluation by Dr. Monteiro-Riviere.

Histologic processing was initiated using MatTek HSE embedded in methylmethacrylate and stained with Masson's trichrome stain. After reviewing the slides, Dr. Pellegrini suggested that routine paraffin-embedding and H&E staining would offer superior detail. We consulted with the COR and MAJ James M. Madsen of USAMRICD and were directed to adopt Dr. Pellegrini's suggestion. ATS HSE, NHS, and a second set of MatTek HSE samples were processed by paraffin embedding and H&E staining and sent to Dr. Pellegrini. The histologic findings based on the assessments of MatTek tissues by Drs. Pellegrini and Monteiro-Riviere were considerably different, perhaps owing to the fact that different lots were examined by each dermatotoxicologist. Thus, after consulting with the COR, new sections of the plastic-embedded MatTek tissue were made, stained with H&E, and assessed by Dr. Pellegrini.

Viability Assessments: The quality of the tissues obtained was screened upon receipt by assessing viability endpoints. Time and resource constraints prevented full assessment of every viability endpoint for each HSE lot and patient skin sample. However, we have compiled an historical database on glucose utilization, lactate production, NAD^+ content, and MTT reduction. Typically, the sample size was four per lot or patient tissue assessed by any one of the viability assays. The methods developed for these assays are briefly described in the following.

Glucose Utilization and Lactate Production Rates: These are nonconsuming metabolic endpoints determined simultaneously with the same instrument, a Model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). They were performed on whole HSE samples and on 5-mm biopsies of NHS. The initial volume of

supplier-specific maintenance medium was 1.0 mL for an ATS HSE sample, and 0.9 mL for a MatTek HSE sample or a NHS biopsy specimen. The medium was changed in the holding well and replaced after 1 hr. This was necessary to remove the accumulation of lactate from overnight incubations. Thereafter, 55-μL aliquots were collected from each specimen well at hourly intervals for 4 hr. The sampled medium was not replaced, but the assay values were corrected for this repeated loss of volume. The samples were analyzed for glucose and lactate concentration simultaneously. The values were regressed against time from 1 to 4 hr after the nutrient medium change to determine slope estimates representing metabolic rates.

NAD$^+$ Content per Biopsy Punch Specimen: Total NAD$^+$ content determinations were performed on 5-mm biopsy punches of ATS HSE and NHS samples, and on 2-mm punches of MatTek HSE samples. Our intent in developing methods for biopsy punch samples was to reduce the cost of performing routine quality control assessments and to make more efficient use of the costly HSEs in subsequent experimental phases. The volume of reagents used depended on the tissue type, but the processes were identical. A biopsy specimen was placed into a centrifuge tube with perchloric acid, refrigerated for 30 min, and then stored at -70 C until assayed. An assay buffering solution of potassium hydroxide and dibasic potassium phosphate was added to the thawed sample, which was vortexed and then refrigerated for 15 min. The samples were centrifuged for 10 min at 1,200 G, and a 40-μL sample was removed and combined with 35 μL of assay buffer in a 96-well microtiter plate well. A 75-μL volume of reaction mix and a 20-μL volume of alcohol dehydrogenase solution (150 U/mL) were added, and the plate was placed into a 37 C incubator for 15 min. A 20-μL volume of a 120-mM sodium iodoacetate solution was added to stop the reaction, and the absorbance was measured in a plate reader at 540 nm. The optical density reading was converted to NAD$^+$ concentration by interpolation on a standard curve. NAD$^+$ concentration was converted to the total NAD$^+$ content in each biopsy specimen. See Attachments 3, 4, and 5 for more details on the NAD$^+$ assay:

- Attachment 3, MREF Method No. 28/In Vitro, Method for the Determination of Nicotinamide Adenine Dinucleotide (NAD$^+$) in Advanced Tissue Sciences (ATS) Skin$^{2™}$ Using the Multiskan MCC 340 MK II ELISA Plate Reader

- Attachment 4, MREF Method No. 29/In Vitro, Method for the Determination of Nicotinamide Adenine Dinucleotide (NAD$^+$) in Natural Human Skin Using the Multiskan MCC 340 MK II ELISA Plate Reader

- Attachment 5, MREF Method No. 30/In Vitro, Method for the Determination of Nicotinamide Adenine Dinucleotide (NAD$^+$) in MatTek Epiderm™ Using the Multiskan MCC 340 MK II ELISA Plate Reader

MTT Reduction: MTT reduction determinations were performed on whole samples and 5-mm biopsy punches of ATS HSE, on whole samples and 2-mm biopsy punches of MatTek HSE, and on 5-mm biopsy punches of split-thickness NHS. Both ATS and MatTek employ the MTT reduction assay as a quality control test for viability on every lot produced. Thus,

each supplier has developed a test method specific to its product. Representative whole samples of tissues received were assayed at the MREF using the supplier's MTT method to determine the effect of shipping on HSE viability upon receipt. By comparing the values determined before and after shipment, the impact of the shipment process could be assessed. In addition, we developed a method for the biopsy punch specimens so that historical databases could be compiled for future quality control purposes and multiple viability endpoints could be performed on a single HSE sample making more economical use of the HSE. For NHS tissue, a method very similar to the ATS 5-mm punch method was used, but extraction of the reduced dye in that case required overnight tissue digestion with Unisol® (Isolab, Inc., Akron, OH). Thus, the MTT assays were similar in procedure but varied in reagent volumes, reaction times, and absorbance wavelength. These MTT assay differences are summarized in Table 1 (Attachment 1).

Basically, the tissue specimen (whole or punch) was placed into a plastic well with a volume of 2 mg/mL MTT in supplier's assay medium, covered, and incubated at approximately 37 C for 2 or 3 hr. The solution was removed and the specimen was washed twice with phosphate buffered saline. The reduced dye was extracted from the cells with either isopropanol (for HSEs) or Unisol® (for NHS), and a sample of the extract was placed into a 96-well plate. Some of the preparations were too concentrated to obtain a reading in the linear portion of the absorbance curve, and these were diluted 1:1 with extraction solution. Absorbance was measured at either 570 nm (whole MatTek samples) or 540 nm (all others). See Attachments 6, 7, and 8 for more details on the MTT assay:

- Attachment 6, MREF Method No. 25/In Vitro, Method for the Measurement of MatTek Epiderm™ Viability Using the MTT Procedure
- Attachment 7, MREF Method No. 26/In Vitro, Method for the Measurement of Advanced Tissue Sciences (ATS) Skin$^{2™}$ Viability Using the MTT Procedure
- Attachment 8, MREF Method No. 27/In Vitro, Method for the Measurement of Natural Human Skin Viability Using the MTT Procedure

Statistical Analysis: Viability data were organized in a database program that was used to sort records by tissue type, specimen size, and test day. The data were loaded into a spreadsheet program that calculated univariate statistics. Slope estimates for glucose utilization and lactate production were calculated by linear regressions against time using the data from all four samples assayed for a given lot. Semi-quantitative histologic findings (e.g., "1+ necrosis") in Dr. Pellegrini's reports were averaged across replicate specimens for a given lot and sample day. Verbal descriptors (e.g., "slight" and severe") in Dr. Monteiro-Riviere's report were converted into semi-quantitative scores and averaged across replicate specimens for a given lot and sample day. The scores ranged from 0 for absence to 5 for severe. When possible, the viability endpoint mean values were compared among themselves and with histologic mean scores.

Results

Histopathology: Mean scores of all histopathologic findings averaged by lot and sample day are presented in Table 2 (Attachment 1). Copies of Dr. Pellegrini's LM reports characterizing the ATS HSE and NHS tissues, and the first and second set of four lots of MatTek HSE tissue, are included as Attachments 9, 10, and 11, respectively. An original of Dr. Monteiro-Riviere's report with glossy photographs characterizing all three tissue types by transmission electron microscopy is included as Attachment 12.

The cover letter of Dr. Pellegrini's first report (Attachment 9) summarized the LM findings for NHS as showing "little deterioration over day 0 to day 7 except for increased perivascular space on day 5 and day 7". This can be seen in Table 2 mean scores, which indicate that there was no observable necrosis in the NHS tissue, perinuclear halos ranged from 1.3 to 2.7, and upper dermal vascular spaces occurred only in Days 5 and 7 specimens. Dr. Monteiro-Riviere's EM report on NHS tissue indicated intercellular epidermal edema that was scored as slight, moderate, or severe in three different Day 0 samples. These observations did not coincide with the upper dermal vascular spaces noted by Dr. Pellegrini. Dr. Monteiro-Riviere concluded (page 17 in Attachment 12) that the overall integrity of the Day 0 NHS was good, but cellular degeneration increased from Days 3 to 7.

Dr. Pellegrini's first report stated that ATS HSE tissue,

> "showed greater variability than human skin but there did not appear to be definitive lot to lot variability. Days 1, 2, and 3 show 1+ necrosis, 1+ sloughing and 1+ clefting when compared to day 0 specimens, though occasionally 1+ changes were noted on day 0 specimens. The only specimens which showed 3 or less cells in thickness of the epidermal layer were day 0 specimens, suggestive of proliferation of epidermal cells with time."

Table 2 presents the data for the above summary, indicating 1+ necrosis in all samples from Lot 1481-111693-138K-7D (7D), and generally no necrosis in the others. Perinuclear halos were generally limited to specimens older than Day 0. The EM report was much more critical of the ATS HSE. In general, when the fibroblast "seed layer appeared healthy, the epidermal cells were not; when the epidermis was healthy, the seed cell layer was inferior." The Day 0 samples exhibited intercellular epidermal edema in three of the four lots, with focal necrosis in two lots and widespread necrosis in two others that were clearly inferior. Referring to these observations, Dr. Monteiro-Riviere concludes,

> "This, in addition to the vacuolization within the stratum corneum and epidermis, the presence of spaces within the dermal matrix, and the discontinuous basement membrane compromised the integrity of this HSE. Utilizing the ATS HSE to study HD dermatotoxicity, which causes these types of effects within normal skin models, would be very difficult."

Cellular degeneration increased in ATS HSE between Days 1 and 3.

Dr. Pellegrini initially reported on the second set of four lots of MatTek HSE, those paraffin-embedded and stained with H&E. His report (Attachment 11) indicated that MatTek HSE consistently exhibited an epidermal thickness in excess of six cell layers on Days 0 and 1. Due to keratinization, this decreased to fewer than six layers on Days 2 and 3 in the lot that was cultured. The keratin layer was generally less than 0.025 mm on Day 0, between 0.025 and 0.05 mm on Day 2, and greater than 0.05 mm on Day 3. Keratinocyte dyskeratosis/necrosis was observed in two Day 0 samples (3+, confluent; or 4+, extensive), was either patchy or absent in the other Day 0 samples and in the Day 1 samples, but completely absent in older samples. Dr. Pellegrini suggested that transport and storage may have been the cause of Day 0 dyskeratinization/necrosis. Nuclear and nucleolar enlargement was observed in all samples, but tended to be less prevalent in Day 0 samples. This second set of MatTek samples was not subjected to E.M. evaluation.

Dr. Pellegrini also examined the first set of four lots of MatTek HSE tissue. These samples were embedded in plastic and initially stained with Masson's trichrome process. The sections were approximately 1 μm thick, and Dr. Pellegrini noted that they were not consistent in a single plane of focus, rendering them difficult to assess. The blocks were resectioned, stained with H&E, and re-examined. Dr. Pellegrini's report on these latter sections (Attachment 10) briefly stated an absence of the following in all samples: necrosis, reactive nucleoli, and keratin layer vacuolization. He noted that as the MatTek HSE tissue specimens aged, epidermal thickness decreased and the keratin layer thickened. All Day 0 samples exhibited more than six epidermal cell layers, and samples from Days 1, 2, and 3 exhibited zero to six cell layers. Day 0 keratin was usually less than 0.025 mm thick, and older samples exhibited keratin usually greater than 0.025 mm thick. In summary, his assessment paralleled Dr. Monteiro-Riviere's regarding these specimens regarding the general absence of necrosis. Thus, the poor condition of the second set of MatTek lots may have had been caused by production problems and/or shipping conditions that were apparently not prevalent in the first set.

Dr. Monteiro-Riviere evaluated specimens from the first four lots of MatTek HSE and reported (Attachment 12) occasional or moderate intercellular epidermal edema in two Day 0 MatTek samples. This model

> "had a poorly developed 'pseudo basement membrane' that lacked a true lamina lucida and lamina densa as well as accompanying anchoring filaments, anchoring fibrils and microfibril bundles. Occasional microvesicles were present along this 'pseudo basement membrane' in Day 2 samples."

Dr. Monteiro-Riviere concluded that the overall integrity of the Day 0 MatTek HSE was good, but that cellular degeneration increased between Days 1 and 3.

The reports generally recommended, for subsequent HD dermatotoxicity studies involving one of the HSE models, that tissues should be used on the day of receipt to avoid increased cellular degeneration in control samples. Dr. Monteiro-Riviere also stressed that control specimens be from the same lot as test specimens in all subsequent studies.

Viability Endpoints: Univariate statistics on glucose utilization and lactate production, NAD^+ content per sample, and MTT reduction as indexed by optical density are presented for ATS HSE, MatTek HSE, and NHS in Tables 3, 4, and 5 (Attachment 1), respectively.

In Table 3, ATS HSE tissue mean glucose utilization rates for whole samples on Day 0 ranged from 0.141 to 0.188 g/L,hr and mean lactate production ranged from 0.0341 to 0.0490 g/L,hr. As shown in Figure 1 (Attachment 2) for the lot abbreviated as 7D, these indices of cell metabolism increased to higher levels on Days 1, 2, and 3, suggesting a surge of tissue growth following the substitution of nutrient agarose with fresh maintenance medium and subsequent incubation. All NAD^+ assays were performed on 5-mm biopsy punches, which exhibited total NAD^+ content on Day 0 ranging from 0.2976 to 0.4826 nmole. This index increased dramatically and remained approximately 3-fold higher across Days 1 to 3 for Lot 7D. Day 0 MTT reduction by 5-mm biopsy punch specimens ranged from an OD_{540} of 0.374 to 0.529. This index appeared to parallel NAD^+ content on Day 0 across lots, but remained at approximately 0.4 for Lot 7D over the three-day time course instead of increasing as did NAD^+ content.

Whole, 11-mm square samples of ATS tissue were also assayed for MTT reduction according to the manufacturer's protocol. The OD_{540} values obtained at the MREF are shown in Table 3 and are presented in Figure 2 (Attachment 2) correlated with values determined by ATS before shipment. In Figure 2, the vertical distance between the line labeled "Slope = 1" and the point for each lot represents the extent of tissue damage incurred during shipment by that lot. The farther below the line that a point is, the more impaired a lot appears to be, according to this metabolic index. Dr. Monteiro-Riviere's evaluations of the epidermis for three lots of ATS HSE tissue are noted in Figure 2 to emphasize her observation that typically the epidermis appeared inferior in conjunction with a healthy and apparently metabolically active fibroblast seed layer (lots abbreviated as 13E and 15B). Lots abbreviated as 17B and 14E15C were not sampled for histologic examination due to their frozen appearance upon receipt. The MTT assay was not performed at the MREF on Lot 7D.

In Table 4 and Figure 3 (Attachment 2), MatTek HSE tissue mean glucose utilization rates for whole samples on Day 0 ranged from 0.206 to 0.254 g/L,hr and mean lactate production ranged from 0.0541 to 0.0793 g/L,hr. These indices generally declined over the three-day time course for Lot 7D, coinciding with the cellular degeneration mentioned by Dr. Monteiro-Riviere. All NAD^+ assays were performed on 2-mm biopsy punches, which exhibited total NAD^+ content on Day 0 ranging from 0.3894 to 0.8517 nmole. In contrast to ATS HSE tissue, MatTek viability in terms of NAD^+ decreased to less than half of the Day 0 level over the time course for Lot 288. Day 0 MTT reduction by 2-mm biopsy punch specimens ranged from an OD_{540} of 0.603 to 1.004. This index appeared to parallel NAD^+ content, decreasing from 1.004 to approximately 0.6 over the three-day time course for Lot 288.

Whole, 9-mm diameter samples were also assayed for MTT reduction according to the manufacturer's protocol. The linear range of the microplate reader was exceeded when the dye extraction solutions were read undiluted. After a 1:1 dilution with extraction solvent, the OD_{540} for the samples ranged from 1.710 to 2.245. Figure 4 (Attachment 2) presents a correlation of the OD_{540} obtained by MatTek before shipping with the OD_{540} obtained at the MREF following receipt. These data indicate a fairly consistent metabolic level as measured by MTT reduction from lot to lot.

In Table 5 the mean glucose utilization rates for 5-mm biopsy specimens of NHS from two patients ranged from 0.057 to 0.209 g/L,hr and mean lactate production ranged from 0.0121 to 0.0196 g/L,hr. By Day 7 these indices of metabolism for Patient No. 93-11-C053 decreased to approximately 75 percent of the Day 0 values, which coincides with the histopathologic assessment. Mean NAD^+ contents on Day 0 were 0.8028 and 1.6972 nmole. This index decreased dramatically in Patient No. 93-11-C053 (abbreviated as C053 in Figure 5, Attachment 2) from 0.8028 to 0.1394 nmole in the 7-day time course. Mean OD_{540}s representing MTT reduction on Day 0 were 1.071 and 1.183. This index decreased in a fashion similar to NAD^+ content over Days 3 to 7 for Patient No. 93-10-C277 (C277 in Figure 5) but remained relatively unchanged for C053.

Discussion

In general, histologic assessments indicated the ATS HSE was less than satisfactory for studying HD dermatotoxicity. Necrosis was prevalent in many samples processed on Day 0, the day of receipt at the MREF. With vacuolization in the epidermis and a discontinuous basement membrane present as background conditions, HD-associated lesions would be difficult to discern in the ATS HSE. Dr. Monteiro-Riviere's observation that the fibroblast seed layer was frequently healthy in appearance when the epidermal layer was inferior (after only 2 or 3 cell layers thick), coupled with our observations on HSE metabolism, i.e.,

- the OD_{540} values for MTT reduction were high relative to manufacturer's preshipment values in two of these "inferior" lots, and
- glucose utilization data appeared to denote a hypermetabolic state,

indicated that the MTT endpoint may not be an accurate index of epidermal health. With a glucose concentration of approximately 4.2 g/L in the nutrient medium, and a metabolic glucose utilization rate of approximately 0.17 g/L/hr, the expected period to glucose depletion would be less than 24 hr which the manufacturer recommends for replacing with fresh maintenance medium. This might explain why tissue samples shipped over a weekend were in a compromised condition on receipt. ATS HSE Lot 7D tissue seemed to improve metabolically after its removal from nutrient agarose and placement into maintenance medium. Indeed, a large portion of Lot 7D tissue appeared to keratinize during the three-day time course, indicating an increase in viability after the nutrient medium was changed.

The MatTek HSE model may be more consistent from lot to lot on receipt, both histologically and metabolically, than ATS HSE. The MatTek HSE seemed to arrive in peak condition, although occasionally somewhat necrotic, and to deteriorate with time. The absence of a fibroblast layer and basement membrane, as well as the limited viability in culture may severely limit the utility of this product in HD pathophysiology studies.

In both HSE models, some Day 0 samples exhibited significant necrosis, whereas others were apparently free of necrosis. This inconsistency in Day 0 samples may require across-the-board histologic confirmation of test sample integrity so that any changes may be attributed to the test regimen in future studies. Examination of the new ATS HSE product may reveal an improvement in interlot tissue consistency.

Split-thickness natural human skin from healthy donors appeared to be fairly consistent from donor to donor by the viability endpoints examined, and was generally reported to be histologically adequate on Days 0 and 3. The most significant limitation to using natural human skin is the supply, i.e., only occasional, approximate 100 cm^2 areas of non-dermatomed skin. Finding a reliable source of large, dermatomed sections of skin would enhance the utility of this *ex vivo* model.

Conditions were established and methods were prepared for performing glucose utilization, lactate production, NAD^+ content, and MTT measurements on biopsy punches from NHS and the two HSEs. These procedures can be used in subsequent studies as a means of making more efficient use of costly and limited product.

The assay method for glucose utilization and lactate production was very simple to perform and seemed to be more sensitive than the MTT assay to the health status of the samples tested. Glucose and lactate data indicated problems with the ATS HSE product while the MTT scores were in line with the manufacturer's preshipment values. For all tissues examined, glucose utilization exceeded lactate production by a factor of at least 2, indicating that aerobic metabolism accounted for less than half of the energy requirements of these tissues.

Recommendations

As all cultures exhibited degradation with time, subsequent experiments conducted under Task 91-24 for the purpose of examining the effects of HD should use the most consistent and freshest tissue possible. Although MatTek HSE offered interlot consistency in terms of metabolism, the absence of an epidermal/dermal interface and limited time of viability in culture limit the utility of this model to assess metabolic indices of HD effects.
Dr. Monteiro-Riviere recommended that ATS HSE not be used unless the supplier can correct the histologic inconsistencies seen. In the initial evaluations of ATS HSE, the material had varying degrees of epidermal necrosis and a discontinuous basement membrane. Since this time, ATS has changed their procedure for growing the product to reflect the

original production procedure. Photomicrographs of an HSE lot provided by ATS indicate an improved product relative to that evaluated under Task 91-24. The basement membrane quality of this newly produced product is not known at the present time. In view of the potential benefits of using a skin tissue model for studying HD pathophysiology, along with the costs involved with developing tissue culture models, we recommend this product be re-evaluated.

NHS could be refrigerated for 3 days before tissue necrosis increased. Dr. Monteiro-Riviere believes a control sample from every tissue lot needs to be concurrently evaluated to assess the effect of HD. Given the small quantity for a given lot received from the Cooperative Human Tissue Network, experimental design will be extremely difficult. A source (Ohio Valley Tissue Center) of square foot quantities of NHS from the same donor and site has been identified. This material is not shipped, however, until serological results are obtained, hence some tissue degradation may occur. It would be worthwhile to perform light microscopic evaluation of this material to evaluate its quality upon receipt. Square foot quantities would aid in experimental designs and make neat HD dosing operations more cost effective. Further efforts should be expended to optimize the NHS *in vitro* model for the purpose of evaluating HD pathophysiology.

Sincerely,

Thomas H. Snider

Thomas H. Snider, B.S., D.A.B.T.
Researcher

THS/tsk

Attachments

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ATTACHMENT 1

Tables

TABLE 1. SUMMARY OF MTT REDUCTION VIABILITY ASSAY FOR VARIOUS HUMAN SKIN TISSUE TYPES AND SIZES

Model System	Human Skin	MatTek Epiderm™		ATS Skin²™ Model ZK1300	
Sample Size	5 mm Biopsy	2 mm Biopsy	Whole	5 mm Biopsy	Whole
MTT concentration:	2 mg/mL	2 mg/mL	2 mg/mL	2 mg/mL	2 mg/mL
Amount of MTT per well:	1 mL	1 mL	300 μL	1 mL	2 mL
Plate size:	24-well	24-well	24-well	24-well	6-well
Incubation Time: 37 C 5% CO_2 ≥ 90% humidity rotation platform	2 hr	2 hr	3 hr	2 hr	2 hr
Wash amount: (PBS Wash Solution)	1 mL	1 mL	Remove insert, gently wash, then place in new 24-well plate with extraction solution.	1 mL	1 mL
Wash time: (Tissue washed twice)	2 min	2 min		2 min	2 min
Extraction amount:	1 mL	125 μL	2 mL	1 mL	4 mL
Extraction solution:	Unisol	Isopropanol	Isopropanol	Isopropanol	Isopropanol
Extraction time: - rotation platform - parafilm plate, cover with lid	Overnight	2 hr	2 hr	1 hr	1 hr
Sample analysis amount: (96 well plate)	1. 100 μL sample 2. 50 μL sample + 50 μL Unisol	80 μL	1. 200 μL 2. 100 μL Sample + 100 μL Isopropanol	100 μL	100 μL Sample + 100 μL Isopropanol
Absorbance:	540 nm	540 nm	570 nm	540 nm	540 nm

TABLE 2. HISTOPATHOLOGY ON TWO HUMAN SKIN EQUIVALENT AND NATURAL HUMAN SKIN TISSUES BY LIGHT AND ELECTRON MICROSCOPY

		Light Microscopy (Dr. Pellegrini)						Electron Microscopy (Dr. Monteiro-Riviere)		
Day	Patient or Lot	Necrosis	Peri-nuclear Halo	Sloughing	Clefting	Upper Dermal Vascular Spaces	Keratin Layer Vacuolization	Inter-cellular Epidermal Edema*	Epidermal Necrosis	General Comment
					Natural Human Skin					
0	93-11-C053	0.0	2.7			0.0		5		
0	93-11-C104R	0.0	1.3			0.0		3		
0	93-10-C253	0.0	1.7			0.0				
0	93-10-C277	0.0	2.7			0.0		2		
3	93-10-C277	0.0	2.3			0.0				
5	93-10-C277	0.0	2.7			0.7				
7	93-10-C277	0.0	2.7			1.0				
					ATS Human Skin Equivalent					
0	1472-091493-138K-20E	0.0	0.0	0.0	0.3			0	focal	
0	1478-102693-138K-13E	0.3	0.0	0.3	1.0			2 to 5	widespread	inferior
0	1481-111693-138K-15B	0.0	0.0	0.0	0.0			2 to 5	widespread	inferior
0	1481-111693-138K-7D	1.0	0.0	0.7	0.3			0 to 3	focal	
1	1481-111693-138K-7D	1.0	1.0	0.3	0.3					
2	1481-111693-138K-7D	1.0	1.0	1.0	0.7					
3	1481-111693-138K-7D	1.0	1.3	0.3	0.3				general	
					MatTek Human Skin Equivalent**					
0	271	0.0								
0	312B	0.0						occasional		
0	312C	0.0							3	
0	311B	0.0								
1	311B	0.0								
2	311B	0.0								
3	311B	0.0								
0	290	2.0					0.0			
0	291	3.3					1.0			
0	424	1.0					0.0			
0	288	0.0					0.0			
1	288	0.0					0.0			
2	288	1.0					1.3			
3	288	1.0					2.0			

* Severity Scores: 0 = none 1 = minimal 2 = slight 3 = moderate 4 = marked 5 = severe

** Dr. Pellegrini did not assess samples #271 through #311B, and Dr. Monteiro-Riviere did not assess samples #290 through #288.

TABLE 3. UNIVARIATE STATISTICS FOR VIABILITY DATA ON ATS HUMAN SKIN EQUIVALENT TISSUE

Test Day	Lot No.	Glucose Utilization (g/L,hr)		Lactate Production (g/L,hr)		NAD^+ Assay				MTT Assay			
						Sample Punch Diam. (mm)	Total NAD^+ (nmole/sample)			Sample Punch Diam. (mm)	Optical Density[a]		
		N	Slope	N	Slope		N	Mean	Std		N	Mean	Std
0	1472-091493-138K-20E	4	0.188	4	0.0490					whole[d]	10	0.696	0.118
0	1477-101993-126K-17B[b]	4	0.166	4	0.0360					whole[d]	3	0.249	0.084
0	1477-101993-138K-14E15C[b]	4	0.175	4	0.0371					whole[d]	3	0.190	0.016
0	1477-101993-138K-13E	4	0.171[c]	4	0.0341[c]					whole[d]	3	0.993	0.095
0	1480-110993-104K-15B	4	0.141[c]	4	0.0370[c]					whole[d]	4	0.706	0.072
0	1481-111693-138K-7D	4	0.175[c]	4	0.0392[c]					whole[d]	9	0.625	0.041
	The following data are also represented in Figure 1.												
1	1472-091493-138K-20E	4	0.258	4	0.0306	5	16	0.7382	0.1582	5	12	0.852	0.157
0	1477-101993-138K-13E	4	0.171	4	0.0341	5	4	0.4342	0.0440	5	4	0.529	0.083
0	1480-110993-104K-15B	4	0.141	4	0.0370	5	4	0.4826	0.1026	5	4	0.399	0.068
0	1481-111693-138K-7D	4	0.175	4	0.0392	5	4	0.2976	0.0214	5	4	0.374	0.026
1	1481-111693-138K-7D	4	0.195	4	0.0769	5	4	1.0951	0.0832	5	4	0.372	0.075
2	1481-111693-138K-7D	4	0.248	4	0.0630	5	4	0.8975	0.2188	5	4	0.440	0.013
3	1481-111693-138K-7D	4	0.227	4	0.0668	5	4	1.0017	0.1113	5	4	0.432	0.124

a = All MTT samples were undiluted unless noted otherwise and read at 540 nm.
b = These lots were judged to be inadequate due to extreme cold during shipping (note the low MTT OD_{540}).
c = This datum is also shown below to complete the table.
d = This MTT sample was diluted 1:1 with isopropanol.

TABLE 4. UNIVARIATE STATISTICS FOR VIABILITY DATA ON MATTEK HUMAN SKIN EQUIVALENT TISSUE

		Glucose Utilization (g/L,hr)		Lactate Production (g/L,hr)		NAD^+ Assay				MTT Assay			
						Sample Punch Diam. (mm)	Total NAD^+ (nmole/sample)			Sample Punch Diam. (mm)	Optical Density		
Day	Lot No.	N	Slope	N	Slope		N	Mean	Std		N	Mean	Std
0	290[a]									whole[b]	4	1.710	0.224
0	291[a]									whole[b]	4	2.245	0.112
0	424[a]									whole[b]	4	1.718	0.066
0	288[a]									whole[b]	4	1.784	0.033
					The following data are also represented in Figure 2.								
0	290	4	0.236	4	0.0694	2	4	0.3938	0.0873	2[c]	4	0.946	0.075
0	291	4	0.206	4	0.0793	2	4	0.3894	0.0139	2[c]	4	0.603	0.034
0	424	4	0.215	4	0.0652	2	4	0.4691	0.0481	2[c]	4	0.917	0.060
0	288	4	0.254	4	0.0541	2	4	0.8517	0.1570	2[c]	4	1.004	0.118
1	288	4	0.202	4	0.0617	2	4	0.3953	0.1666	2[c]	4	0.610	0.073
2	288	4	0.191	4	0.0453	2	4	0.3972	0.2001	2[c]	4	0.629	0.027
3	288	4	0.155	4	0.0356	2	4	0.3127	0.1478	2[c]	4	0.615	0.033

a = Only the MTT assay was performed on whole tissue samples of MatTek HSE.
b = MTT samples were diluted 1:1 with isopropanol and read at 570 nm.
c = MTT samples from 2-mm punches were undiluted and read at 540 nm.

TABLE 5. UNIVARIATE STATISTICS FOR VIABILITY DATA ON NATURAL HUMAN SKIN TISSUE

Test Day	Patient No.	Glucose Utilization (g/L,hr)		Lactate Production (g/L,hr)		NAD+ Assay				MTT Assay			
						Sample Punch Diam. (mm)	Total NAD+ (nmole/sample)			Sample Punch Diam. (mm)	Optical Density[a]		
		N	Slope	N	Slope		N	Mean	Std		N	Mean	Std
0	93-11-C104R	4	0.209	4	0.0121	5	3	1.6972	0.2780	5[b]	4	1.071	0.118
0	93-11-C053	4	0.057	4	0.0196	5	4	0.8028	0.0671	5[b]	4	1.183	0.226
3	93-11-C053	4	0.064	4	0.0155	5	4	0.2771	0.1084	5[b]	4	1.109	0.164
5	93-11-C053	4	0.058	4	0.0168	5	4	0.1935	0.1065	5[b]	4	1.283	0.087
7	93-11-C053	4	0.044	4	0.0147	5	4	0.1394	0.0876	5[b]	4	0.761	0.096
0	93-11-C104R[c]									5[d]	4	0.553	0.052
0	93-11-C053[c]									5[d]	4	0.601	0.132
3	93-11-C053[c]									5[d]	4	0.560	0.078
5	93-11-C053[c]									5[d]	4	0.650	0.091
7	93-11-C053[c]									5[d]	4	0.418	0.063

a = All MTT samples were read at 540 nm.
b = MTT samples were undiluted.
c = These entries were included to show the effect of diluting with tissue solubilizer.
d = MTT samples were diluted 1:1 with Unisol®.

ATTACHMENT 2

Figures

FIGURE 1. ATS HUMAN SKIN EQUIVALENT VIABILITY, 5-MM SPECIMENS

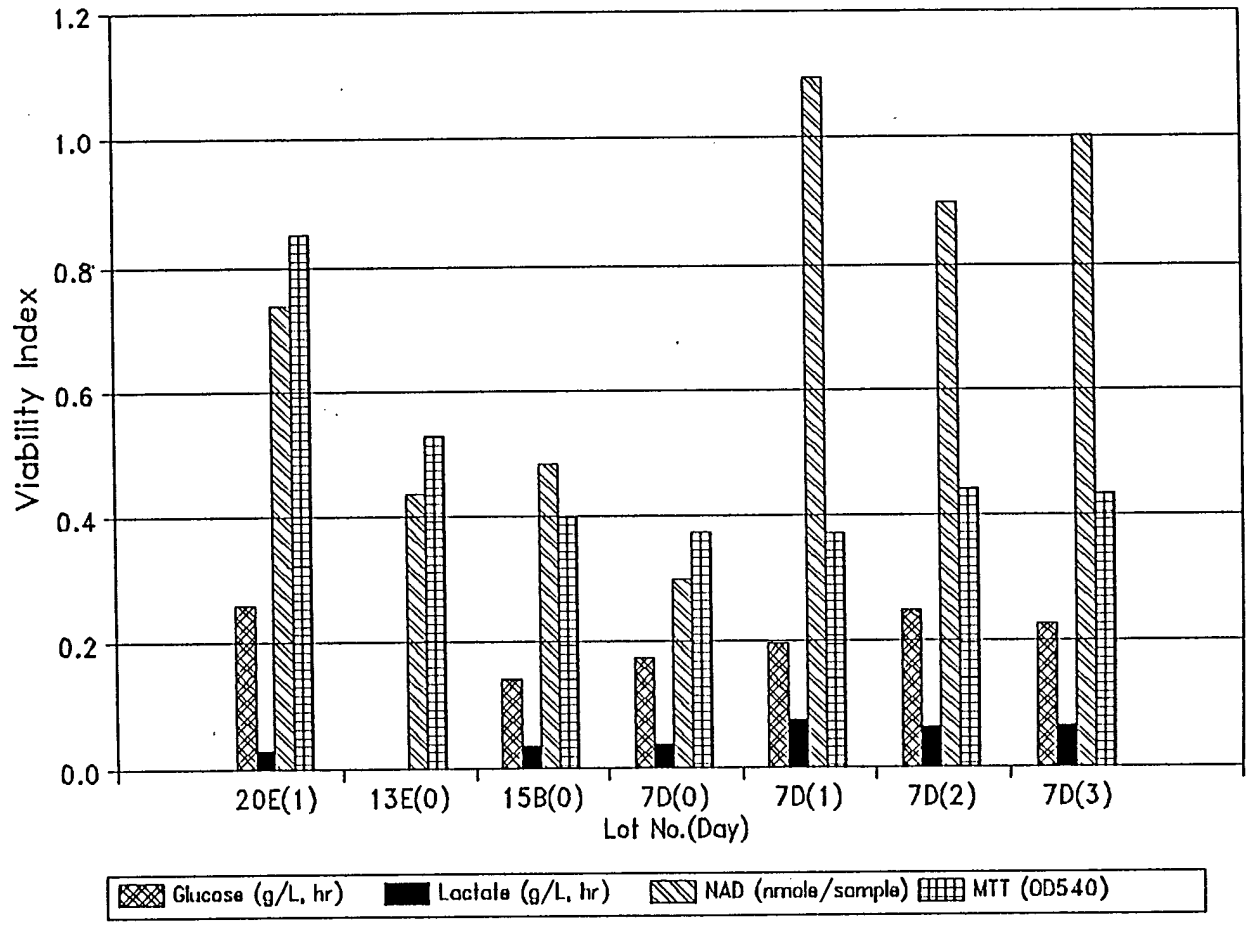


FIGURE 2. EFFECT OF SHIPMENT ON WHOLE SAMPLES OF ATS HUMAN SKIN EQUIVALENT TISSUE

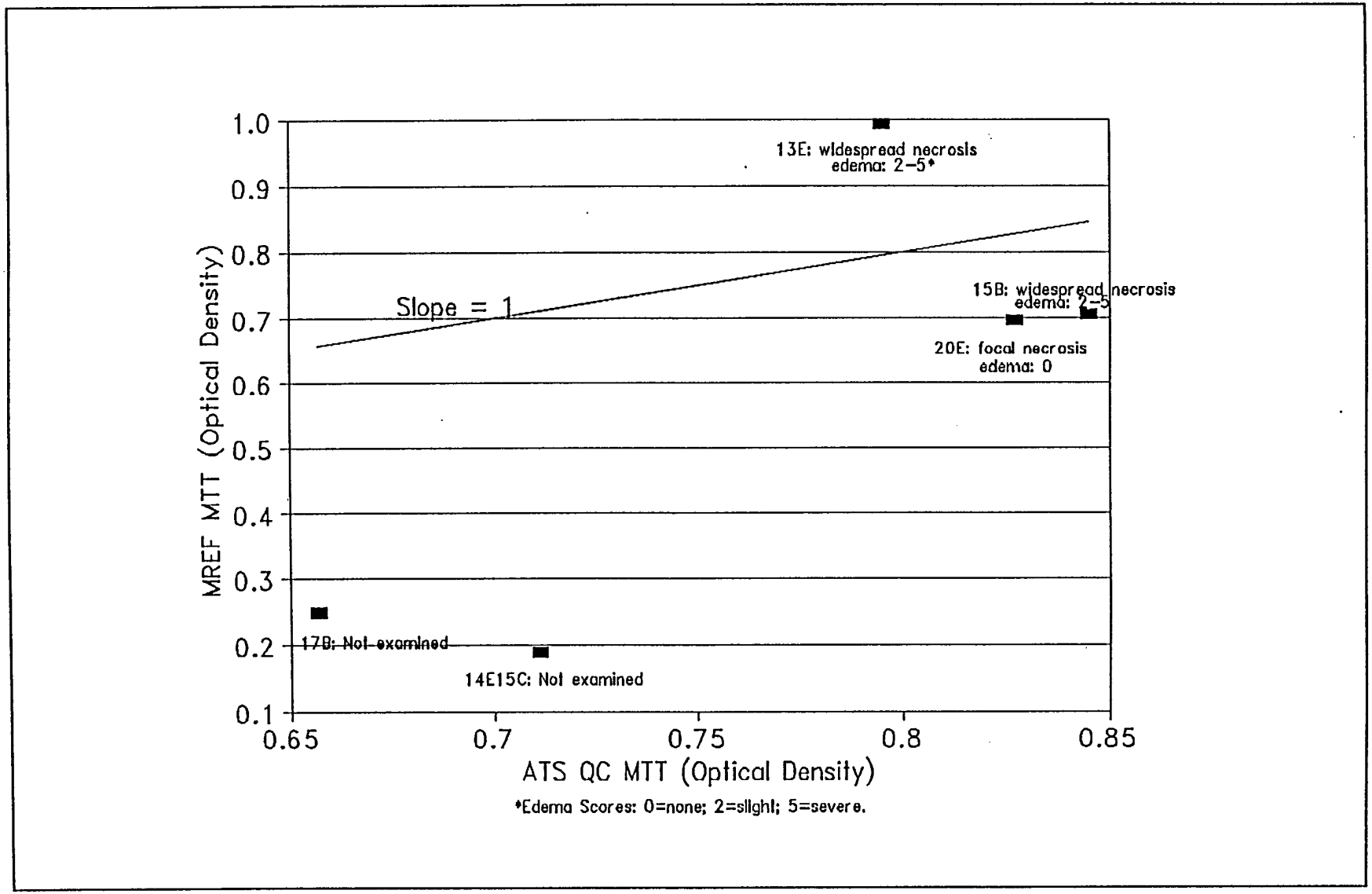


FIGURE 3. MATTEK HUMAN SKIN EQUIVALENT VIABILITY, 2-MM SPECIMENS

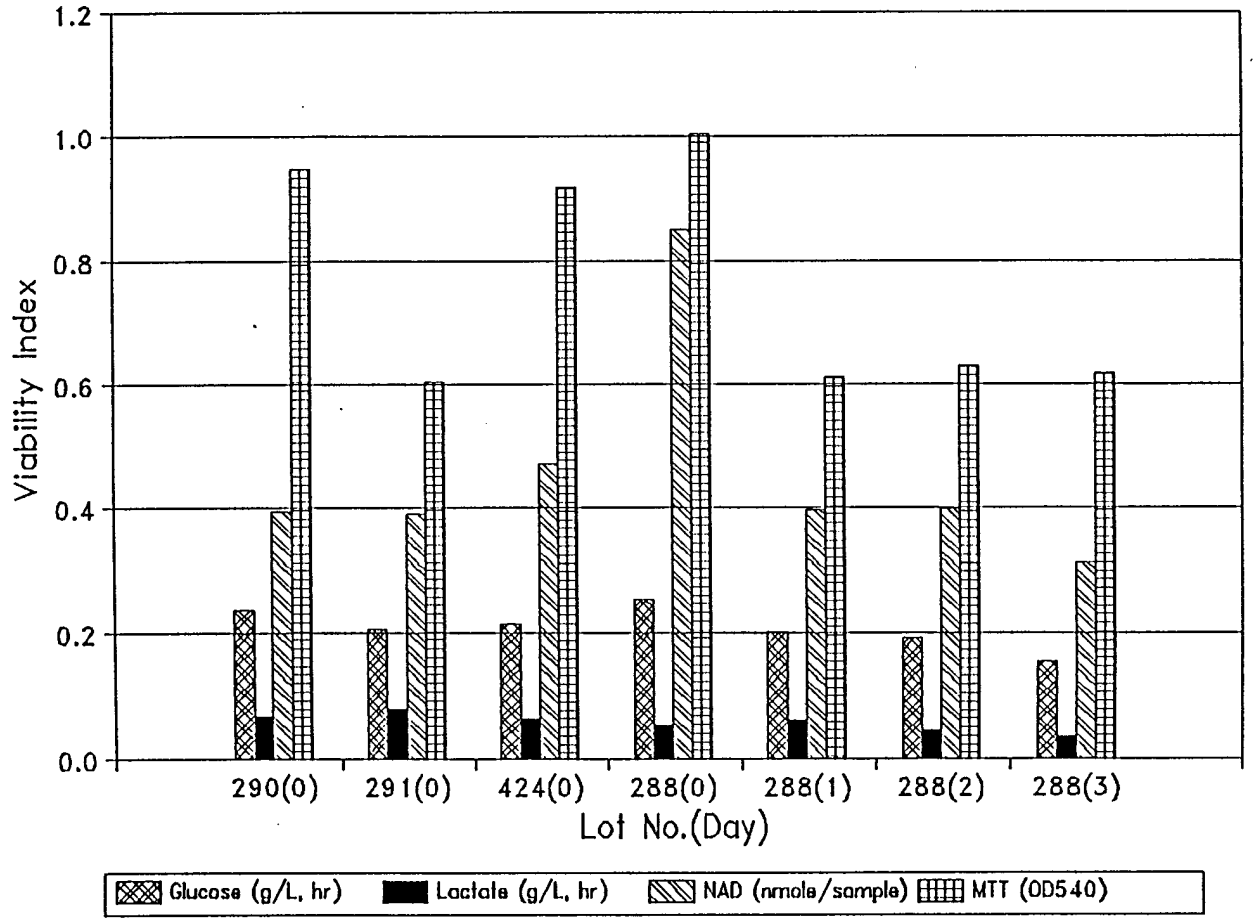


FIGURE 4. EFFECT OF SHIPMENT ON WHOLE SAMPLES OF MATTEK HUMAN SKIN EQUIVALENT SPECIMENS

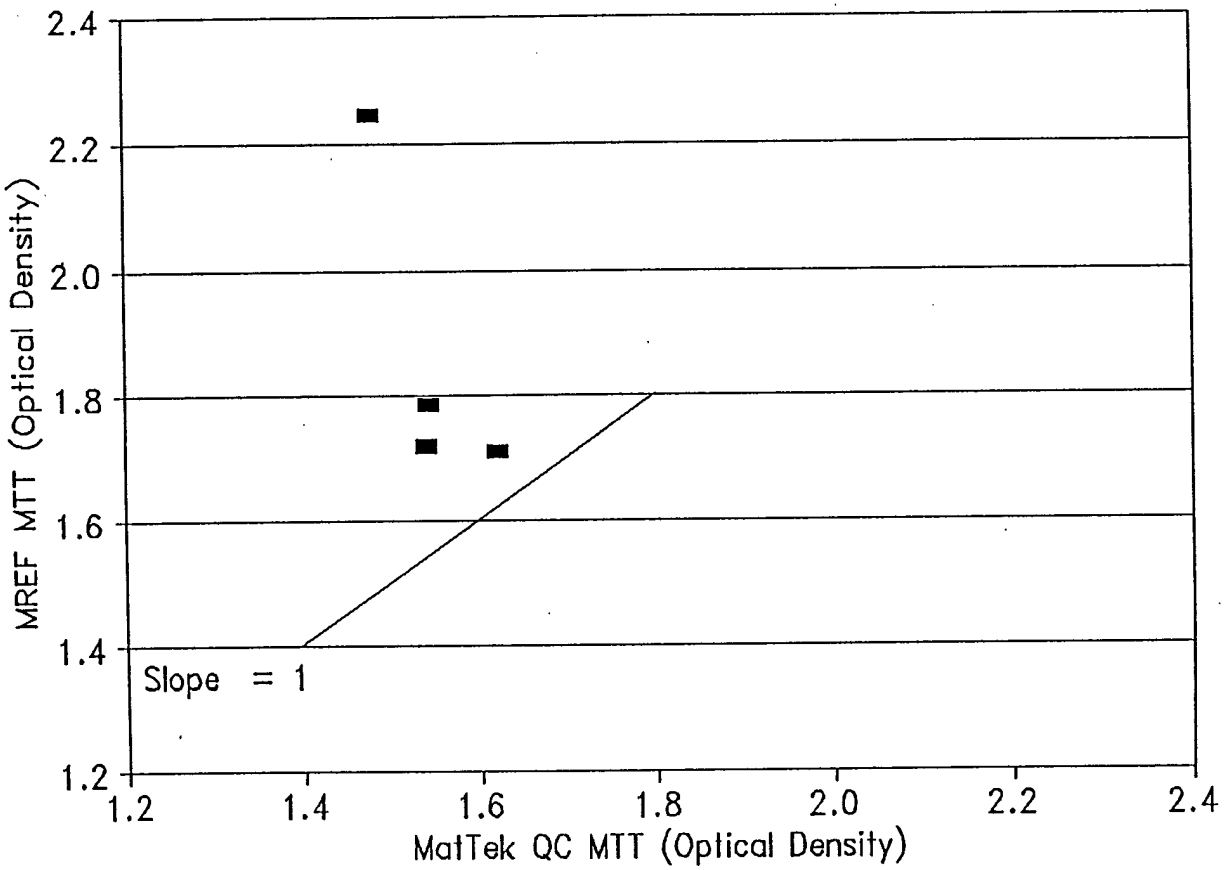
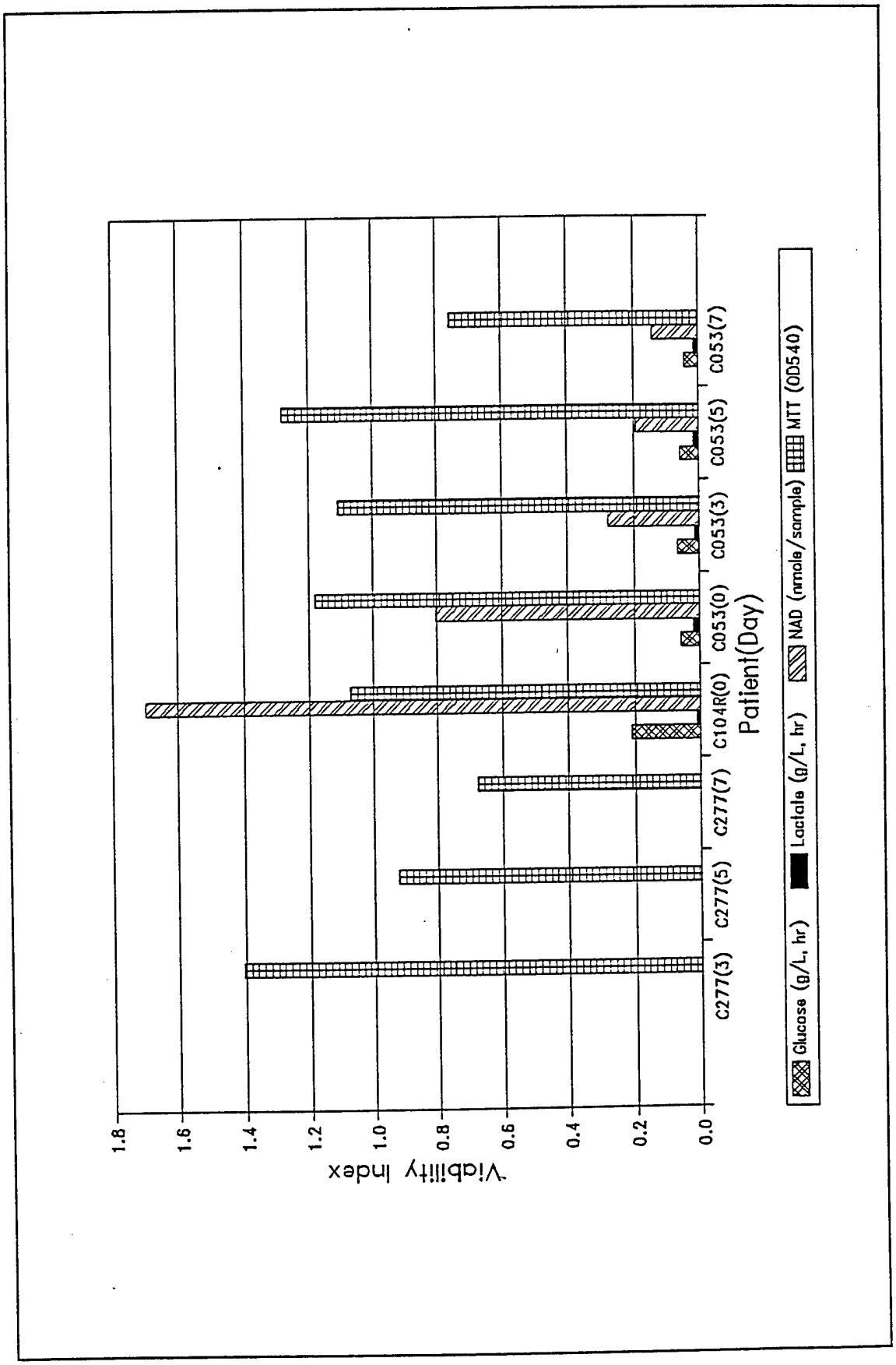


FIGURE 5. NATURAL HUMAN SKIN VIABILITY, 5-MM SPECIMENS



ATTACHMENT 3

MREF Method No. 28/In Vitro

METHOD FOR THE DETERMINATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) IN ADVANCED TISSUE SCIENCES (ATS) SKIN^{2TM} USING THE MULTISKAN MCC 340 MK II ELISA PLATE READER

A. Statement of Work: This method describes the procedures used to measure nicotinamide adenine dinucleotide (NAD^+) levels in ATS Skin^{2TM} samples. This procedure described in this method for NAD^+ analysis is based upon that of Jacobson and Jacobson (Section H.3.) adapted for use in a 96 well microtiter plate system.

B. Abbreviations and Reagents:

1. ADH - Alcohol Dehydrogenase (Sigma #A3263; 280 U/mg solid)
2. BSA - Bovine Serum Albumin
3. EDTA - Ethylenediamine tetraacetic acid
4. EtOH - Absolute ethanol
5. HCl - Hydrochloric Acid
6. $HClO_4$ - Perchloric Acid
7. K_2HPO_4 - dibasic Potassium Phosphate
8. KOH - Potassium Hydroxide
9. MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
10. NAD^+ - Nicotinamide Adenine Dinucleotide (oxidized)
11. PES - Phenazine ethosulfate
12. Bicine - N, N-Bis(2-hydroxyethyl)glycine

C. Precautions:

1. $HClO_4$ - $HClO_4$ is a potent oxidizing and caustic agent. Its use in the presence of organic solvents must be avoided. Dilutions of stock $HClO_4$ (60 - 70 percent $HClO_4$ solution) must be made in the biological safety cabinet as should all other work with diluted $HClO_4$ solution.

2. MTT and PES - These are labeled as being suspect carcinogens. They should be treated as such and weighed on a scale located in a vented cabinet. These are also light sensitive chemicals and should be weighed under reduced lighting. The container in which they are solubilized should be enclosed in foil.

D. Preparation of Reagents:

1. $HClO_4$ Dilution - All dilutions and additions of $HClO_4$ are to be performed in a biological safety cabinet that does not contain any organic solvents. The procedure requires two individuals. $HClO_4$ can be obtained as an approximate 60 to 70 percent solution in water. The stock bottle is kept in a secondary container stored in a cabinet that is void of any organic acids or solvents. The molarity of the stock $HClO_4$ solution is calculated as follows:

a. $$\frac{(1.768\ \text{gm/mL}) \times (1{,}000\ \text{mL/L})}{100.42\ \text{gm/mol}} = X\text{mol/L}$$

b. X multiplied by the percent of the $HClO_4$ solution and divided by 100 gives the molarity of the stock $HClO_4$ solution.

Prepare a 3 M $HClO_4$ solution in the biological safety cabinet over plastic backed paper using distilled water as the diluent. The solution is not used 60 days past the date of preparation.

c. One individual will remove the $HClO_4$ from the secondary container, open the cap of the $HClO_4$, and recap the bottle as soon as the aliquot is removed. The second individual removes and transfers the volume of stock $HClO_4$ to the Erlenmeyer flask. The $HClO_4$ is dispensed below the level of the distilled water. Just after dispensing, the serological pipette is filled with the diluted acid solution and this material is dispensed back into the Erlenmeyer flask. The Erlenmeyer is stoppered and clearly labeled with the following information: contents, molarity, date of preparation, and individual making the solution. The 3 M $HClO_4$ solution is kept refrigerated and will not be used 60 days past the date of preparation. Prepare 0.5 m $HClO_4$ by adding 30 mL of 3 M $HClO_4$ to 150 mL of distilled deionized water. The first individual replaces the $HClO_4$ in its secondary container and returns the secondary container to the acid cabinet.

2. Preparation of KOH/K_2HPO_4 Buffer:

a. Dissolve 28.74 gm of K_2HPO_4 in 400 mL of distilled water contained in a 500-mL beaker and adjust the pH to 7.8 with 3 N HCl.

b. Dissolve 28.05 gm of KOH in the phosphate buffered solution prepared above in Section 2.a., transfer the solution to a 500-mL volumetric flask, and fill to the 500 mL volume mark with distilled water. The solution contains 0.33 M K_2HPO_4 and 1 M KOH. The container will be labeled identifying the contents of the container, date of preparation, and the initials of the individual making the solution.

c. The KOH/K_2HPO_4 buffer is used to neutralize the $HClO_4$ solution. As slight variation in the $HClO_4$ and KOH/K_2HPO_4 solution may exist when the solutions are prepared, it is necessary to titrate the $HClO_4$ solution with KOH/K_2HPO_4 when either of the solutions is prepared. A 12 mL volume of 0.5 m $HClO_4$ is added to a 20 mL scintillation vial. A 5 mL volume of KOH/K_2HPO_4 buffer is added to the $HClO_4$, the solution mixed and the solution pH determined. Aliquots of 50 µL of KOH/K_2HPO_4 buffer is added to the scintillation vial until the resulting solution pH is between 6.8

and 7.0. The cumulative or total volume (mL) of KOH/K_2HPO_4 buffer required to achieve this final pH (6.8 to 7.0) is computed. Dividing this value by 100 yields the volume in microliters of KOH/K_2HPO_4 required to neutralize 120 µL of 0.5 M $HClO_4$ solution. This volume should be 60 µL ± 5 µL. This volume, along with the date, is marked on the $HClO_4$ and KOH/K_2HPO_4 containers. If outside this range, either the $HClO_4$ or the KOH/K_2HPO_4 solutions should be prepared.

d. The solution is kept refrigerated and will not be used 60 days past the date of preparation.

3. Preparation of Assay Buffers:

a. Buffer I:

Reagent	Concentration	g/100 mL
Bicine	200 mM	3.264
EDTA	8.32 mM	0.310
BSA	1.66 mg/mL	0.166

Adjust to pH 7.8 with 1N KOH. Mark date of preparation, initial of individual preparing solution, and the expiration date which is 2 months when stored refrigerated.

b. Buffer II:

Reagent	Concentration	g/20 mL
MTT	0.84 mM	0.0068
PES	6.64 mM	0.0444
EtOH	500.00 mM	

(1) Prepare just prior to use. Dissolve the MTT and PES in 18.8 mL of Buffer I. Then add 1.2 mL of EtOH and mix. This is performed under reduced lighting.

(2) Wrap the container with aluminum foil immediately after preparation to protect from lighting. Discard solution after use.

4. Preparation of ADH: Prepare using distilled deionized water on the day of use and store refrigerated. Add 0.004 gm of ADH to 7.5 mL of diluent to yield a 150 U/mL solution. Discard solution after use.

5. Preparation of NAD^+ Standards:

a. 1.5×10^{-3} M Stock: Add 0.0054 gm of NAD^+ to 5 mL of 0.5 M $HClO_4$ and mix. Use 0.5 M $HClO_4$ as the diluent in subsequent dilutions.

b. 1.5×10^{-5} M (Working Stock): Add 0.05 mL of a. to 5 mL of diluent and mix.

c. 1.13×10^{-6} M: 0.75 mL of b. + 9.25 mL diluent.

d. 7.5×10^{-7} M: 4 mL of c. + 2 mL diluent.

e. 3.75×10^{-7} M: 3 mL of d. + 3 mL diluent.

f. 1.88×10^{-7} M: 3 mL of e. + 3 mL diluent.

g. 9.4×10^{-8} M: 3 mL of f. + 3 mL diluent.

h. 4.7×10^{-8} M: 3 mL of g. + 3 mL diluent.

i. Background: 0.5 M perchloric acid used for diluent.

Prepare the dilutions of the standards and place 0.5-mL aliquots into a series of Eppendorf centrifuge tubes. The tubes should be labeled indicating the standard letter, date of preparation, and individual preparing the dilution. The standards can be used for up to 1 month following preparation when stored frozen.

6. Preparation of Sodium Iodoacetate:

A 120 mM solution of sodium iodoacetate is prepared by adding 1.248 gm to 50 mL of distilled water. The solution container is marked to indicate contents, a 30-day expiration date, and the name of the individual preparing the solution.

E. Instrument Power Up:

1. Turn on the ELISA reader.

2. Press Measurement Mode - press arrow key until "single wavelength absorbance" appears on screen and press enter.

3. Using the numerical keys, press 540 and enter.

4. Press arrow key until "no background" appears on screen and press enter.

5. Insert the microtiter plate onto the reader platform and press start. The Titertek will print out the absorbance values. This printout will be appropriately identified, initialed, and dated. The printout must be photo copied for the study records, as the printout will fade with time.

6. Perform assays with the standards first to determine a standard curve, and to ensure that no problems exist with the reagents. Then proceed to assay the samples.

F. Extraction of NAD^+ from ATS Skin^{2TM}:

1. Place a 5-mm biopsy of the ATS Skin^{2TM} sample into a centrifuge tube containing 1.0 mL of $HClO_4$ and cap the centrifuge tube. Repeat the process for each 5-mm biopsy collected for an NAD^+ assay. Refrigerate the samples for 30 min. Transfer the samples to the -70 C freezer until further analysis.

2. Transfer the frozen ATS Skin^{2TM} samples and the container of KOH/K_2HPO_4 to a sterile hood. Thaw the ATS Skin^{2TM} samples. Determine the volume of KOH/K_2HPO_4 needed to titrate ATS Skin^{2TM} samples. Titration is determined in Section D.2.c. Add KOH/K_2HPO_4 to the samples, and this final volume is called "total sample volume". Replace the lids on the centrifuge tubes and vortex the samples. Place the ATS Skin^{2TM} samples in the refrigerator for a minimum of 15 min.

3. Centrifuge the samples at 1,200 x g (2,500 rpm) for 10 min.

G. Preparation of "Buffer Volume" Solution:

1. Calculate the amount of "buffer volume" solution required for all NAD^+ samples. This is calculated by multiplying the total number of samples by 35 µL. Prepare an additional 3 mL more of "buffer volume" solution than is actually required. "Buffer volume" solution is prepared by titrating 0.5 M $HClO_4$ with KOH/K_2HPO_4, as determined in section D.2.c. After adding KOH/K_2HPO_4 to the $HClO_4$, vortex and place the solution into the refrigerator for a minimum of 15 min. Then centrifuge the "buffer volume" solution at 1,200 x g (2,500 rpm) for 10 min.

H. Analysis of NAD^+:

1. Thaw a set of NAD^+ standards, add 250 µL of the KOH/K_2HPO_4 solution, vortex, then incubate in the refrigerator for 15 min. Centrifuge the standards for 10 min at 1,200 x g (2,500 rpm).

2. Add 75 µL of background (0 nM NAD^+ standard) and NAD^+ standards to each of four wells. This yields standards with NAD^+ levels approximately 750, 500, 250, 125, 62.5, 31.25, and 0 nM for standards described in Sections E.5.c. to E.5.h., respectively. Assay the background and standards on a plate separate from the samples. When the samples are assayed, transfer and combine 40 µL of sample (the "partial sample volume") with 35 µL of assay buffer (the "buffer volume") to a new 96-well microtiter plate for a "total diluent volume" of 75 µL. Include 75 µL of "buffer volume" with samples for background analysis. Record sample positions on Attachment #1.

3. Add 75 µL of Buffer II to each sample well under dimmed lights.

4. Add 20 µL of ADH (150 U/mL) under dimmed lights to initiate the reaction. Cover the 96-well plate with a lid and place into the incubator for 15 min.

5. After 15 min, promptly add 20 µL of sodium iodoacetate (120 mM) solution to each sample well. (Be sure to wipe the bottom of the plate thoroughly with lens paper to remove any condensation.

6. Immediately measure the absorbance of the wells at 540 nm using a Titertek Multiskan MCC/340 ELISA plate reader following Battelle SOP MREF V-002.

I. Calculation of NAD^+:

1. Regression analysis is performed on the standards to obtain slope (m) and y-intercept (b) values. Nanomolar concentrations of NAD^+ standards are the independent (x) variable, and background-subtracted absorbance values at 540 nm are the dependent (y) variable.

2. Background-subtracted absorbance values at 540 nm for samples are converted to picomoles of NAD^+ per sample using the following series of calculations:

(a) Line equation $x = (y - b)/m$

where: m = slope
b = y-intercept
y = background-subtracted absorbance at 540 nm
x = $[NAD^+]$ (nM)

(b) Total NAD^+ (pmoles) = $[NAD^+]$ (nM) * Total diluent volume (µL) *

$$\frac{\text{Total sample volume (µL)}}{\text{partial sample volume(µL)}} * \frac{1000 \text{ pmoles}}{\text{nmole}} * \frac{1\text{L}}{10^6 \mu\text{L}}$$

J. References:

1. Mol, M.A.E., A.B.C. Van De Ruit, and A.W. Kluivers, NAD^+ Levels and Glucose Uptake of Cultured Human Epidermal Cells Exposed to Sulfur Mustard, Tox. App. Pharmacol. 98:159-165 (1989).

2. Meier, H. L., C.L. Gross, and B. Papirmeister, 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard) Decreases NAD^+ Levels in Human Leukocytes, Tox. Lett. 39:109-122 (1987).

3. Jacobson, E.L. and M.K. Jacobson, Pyridine Nucleotide Levels as a Function of Growth in Normal and Transformed 3T3 Cells, Arch. Biochem. Biophys. 175:627-634 (1976).

4. Blank, J., Method for the Determination of Nicotinamide Adenine Dincleotide (NAD^+) Using the Multiskan MCC 340 MK II Elisa Plate Reader, Method #1/In Vitro-01. (1993)

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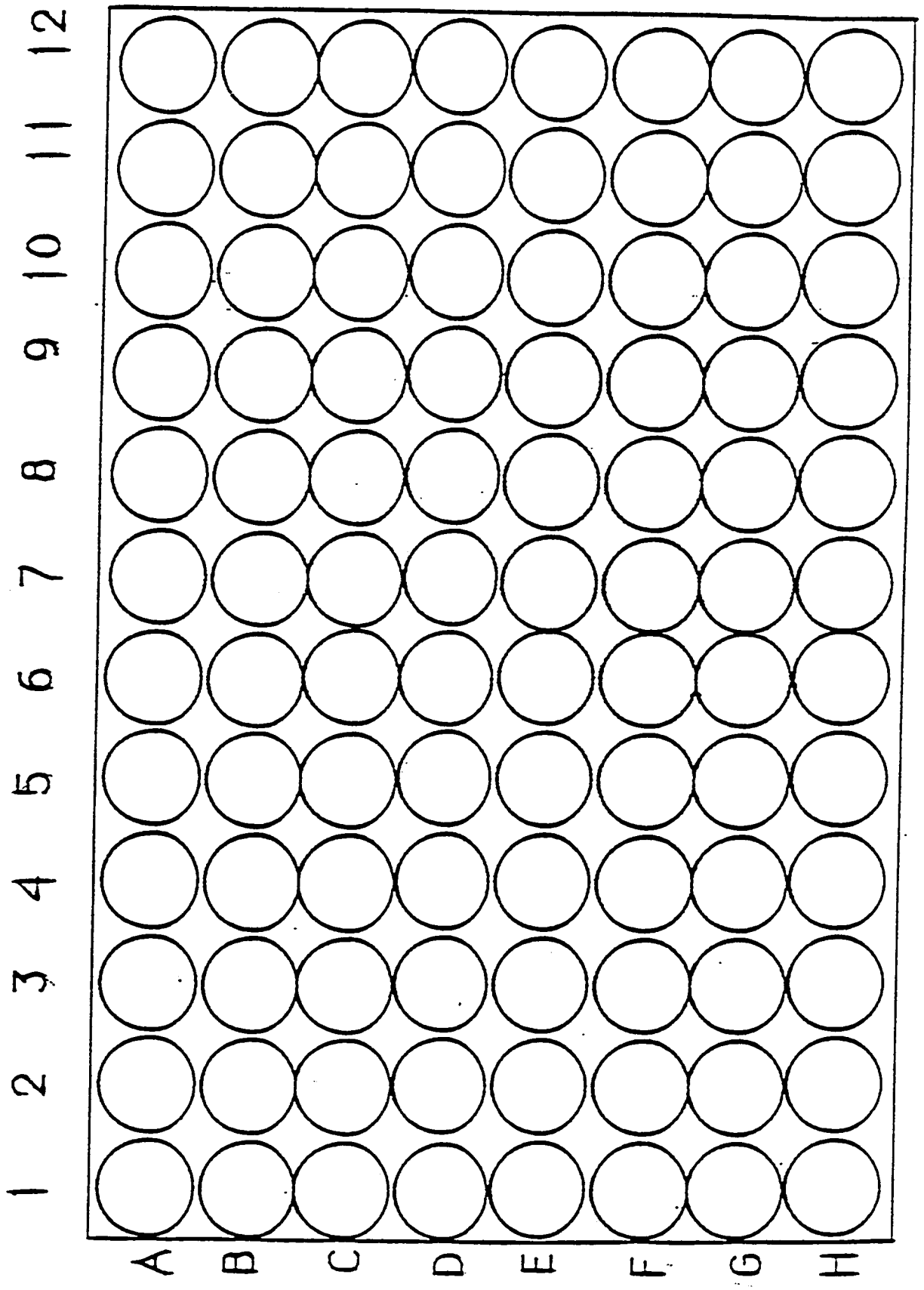
Date: 4/11/94

Reviewed by: Thomas, H. Snider, B.S.
Research Scientist
Date: 4/11/94

ATTACHMENT #1



ATTACHMENT 4

MREF Method No. 29/In Vitro

METHOD FOR THE DETERMINATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) IN NATURAL HUMAN SKIN USING THE MULTISKAN MCC 340 MK II ELISA PLATE READER

A. Statement of Work: This method describes the procedures used to measure nicotinamide adenine dinucleotide (NAD^+) levels in natural human skin samples. This procedure described in this method for NAD^+ analysis is based upon that of Jacobson and Jacobson (Section H.3.) adapted for use in a 96 well microtiter plate system.

B. Abbreviations and Reagents:

1. ADH - Alcohol Dehydrogenase (Sigma #A3263; 280 U/mg solid)
2. BSA - Bovine Serum Albumin
3. EDTA - Ethylenediamine tetraacetic acid
4. EtOH - Absolute ethanol
5. HCl - Hydrochloric Acid
6. $HClO_4$ - Perchloric Acid
7. K_2HPO_4 - Potassium Phosphate
8. KOH - Potassium Hydroxide
9. MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
10. NAD^+ - Nicotinamide Adenine Dinucleotide (oxidized)
11. PES - Phenazine ethosulfate
12. Bicine - N, N-Bis(2-hydroxyethyl)glycine

C. Precautions:

1. $HClO_4$ - $HClO_4$ is a potent oxidizing and caustic agent. Its use in the presence of organic solvents must be avoided. Dilutions of stock $HClO_4$ (60 - 70 percent $HClO_4$ solution) must be made in the biological safety cabinet as should all other work with diluted $HClO_4$ solution.

2. MTT and PES - These are labeled as being suspect carcinogens. They should be treated as such and weighed on a scale located in a vented cabinet. These are also light sensitive chemicals and should be weighed under reduced lighting. The container in which they are solubilized should be enclosed in foil.

D. Preparation of Reagents:

1. $HClO_4$ Dilution - All dilutions and additions of $HClO_4$ are to be performed in a biological safety cabinet that does not contain any organic solvents. The procedure requires two individuals. $HClO_4$ can be obtained as an approximate 60 to 70 percent solution in water. The stock bottle is kept in a secondary container stored in a cabinet that is void of any organic acids or solvents. The molarity of the stock $HClO_4$ solution is calculated as follows:

a. $$\frac{(1.768\ gm/mL) \times (1{,}000\ mL/L)}{100.42\ gm/mol} = X mol/L$$

b. X multiplied by the percent of the $HClO_4$ solution and divided by 100 gives the molarity of the stock $HClO_4$ solution.

Prepare a 3 M $HClO_4$ solution in the biological safety cabinet over plastic backed paper using distilled water as the diluent. The solution is not used 60 days past the date of preparation.

c. One individual will remove the $HClO_4$ from the secondary container, open the cap of the $HClO_4$, and recap the bottle as soon as the aliquot is removed. The second individual removes and transfers the volume of stock $HClO_4$ to the Erlenmeyer flask. The $HClO_4$ is dispensed below the level of the distilled water. Just after dispensing, the serological pipette is filled with the diluted acid solution and this material is dispensed back into the Erlenmeyer flask. The Erlenmeyer is stoppered and clearly labeled with the following information: contents, molarity, date of preparation, and individual making the solution. The 3 M $HClO_4$ solution is kept refrigerated and will not be used 60 days past the date of preparation. Prepare 0.5 m $HClO_4$ by adding 30 mL of 3 M $HClO_4$ to 150 mL of distilled deionized water. The first individual replaces the $HClO_4$ in its secondary container and returns the secondary container to the acid cabinet.

2. Preparation of KOH/K_2HPO_4 Buffer:

a. Dissolve 28.74 gm of K_2HPO_4 in 400 mL of distilled water contained in a 500-mL beaker and adjust the pH to 7.8 with 3 N HCl.

b. Dissolve 28.05 gm of KOH in the phosphate buffered solution prepared above in Section 2.a., transfer the solution to a 500-mL volumetric flask, and fill to the 500 mL volume mark with distilled water. The solution contains 0.33 M K_2HPO_4 and 1 M KOH. The container will be labeled identifying the contents of the container, date of preparation, and the initials of the individual making the solution.

c. The KOH/K_2HPO_4 buffer is used to neutralize the $HClO_4$ solution. As slight variation in the $HClO_4$ and KOH/K_2HPO_4 solution may exist when the solutions are prepared, it is necessary to titrate the $HClO_4$ solution with KOH/K_2HPO_4 when either of the solutions is prepared. A 12 mL volume of 0.5 m $HClO_4$ is added to a 20 mL scintillation vial. A 5 mL volume of KOH/K_2HPO_4 buffer is added to the $HClO_4$, the solution mixed and the solution pH determined. Aliquots of 50 μL of KOH/K_2HPO_4 buffer is added to the scintillation vial until the resulting solution pH is between 6.8 and 7.0. The cumulative or total volume (mL) of KOH/K_2HPO_4 buffer

required to achieve this final pH (6.8 to 7.0) is computed. Dividing this value by 100 yields the volume in microliters of KOH/K_2HPO_4 required to neutralize 120 µL of 0.5 M $HClO_4$ solution. This volume should be 60 µL ± 5 µL. This volume, along with the date, is marked on the $HClO_4$ and KOH/K_2HPO_4 containers. If outside this range, either the $HClO_4$ or the KOH/K_2HPO_4 solutions should be prepared.

d. The solution is kept refrigerated and will not be used 60 days past the date of preparation.

3. Preparation of Assay Buffers:

a. Buffer I:

Reagent	Concentration	g/100 mL
Bicine	200 mM	3.264
EDTA	8.32 mM	0.310
BSA	1.66 mg/mL	0.166

Adjust to pH 7.8 with 1N KOH. Mark date of preparation, initial of individual preparing solution, and the expiration date which is 2 months when stored refrigerated.

b. Buffer II:

Reagent	Concentration	g/20 mL
MTT	0.84 mM	0.0068
PES	6.64 mM	0.0444
EtOH	500.00 mM	

(1) Prepare just prior to use. Dissolve the MTT and PES in 18.8 mL of Buffer I. Then add 1.2 mL of EtOH and mix. This is performed under reduced lighting.

(2) Wrap the container with aluminum foil immediately after preparation to protect from lighting. Discard solution after use.

4. Preparation of ADH: Prepare using distilled deionized water on the day of use and store refrigerated. Add 0.004 gm of ADH to 7.5 mL of diluent to yield a 150 U/mL solution. Discard solution after use.

5. Preparation of NAD^+ Standards:

a. 1.5×10^{-3} M Stock: Add 0.0054 gm of NAD^+ to 5 mL of 0.5 M $HClO_4$ and mix. Use 0.5 M $HClO_4$ as the diluent in subsequent dilutions.

b. 1.5×10^{-5} M (Working Stock): Add 0.05 mL of a. to 5 mL of diluent and mix.

c. 1.13×10^{-6} M: 0.75 mL of b. + 9.25 mL diluent.

d. 7.5×10^{-7} M: 4 mL of c. + 2 mL diluent.

e. 3.75×10^{-7} M: 3 mL of d. + 3 mL diluent.

f. 1.88×10^{-7} M: 3 mL of e. + 3 mL diluent.

g. 9.4×10^{-8} M: 3 mL of f. + 3 mL diluent.

h. 4.7×10^{-8} M: 3 mL of g. + 3 mL diluent.

i. Background: 0.5 M perchloric acid used for diluent.

Prepare the dilutions of the standards and place 0.5-mL aliquots into a series of Eppendorf centrifuge tubes. The tubes should be labeled indicating the standard letter, date of preparation, and individual preparing the dilution. The standards can be used for up to 1 month following preparation when stored frozen.

6. Preparation of Sodium Iodoacetate:

A 120 mM solution of sodium iodoacetate is prepared by adding 1.248 gm to 50 mL of distilled water. The solution container is marked to indicate contents, a 30-day expiration date, and the name of the individual preparing the solution.

E. Instrument Power Up:

1. Turn on the ELISA reader.

2. Press Measurement Mode - press arrow key until "single wavelength absorbance" appears on screen and press enter.

3. Using the numerical keys, press 540 and enter.

4. Press arrow key until "no background" appears on screen and press enter.

5. Insert the microtiter plate onto the reader platform and press start. The Titertek will print out the absorbance values. This printout will be appropriately identified, initialed, and dated. The printout must be photo copied for the study records, as the printout will fade with time.

6. Perform assays with the standards first to determine a standard curve, and to ensure that no problems exist with the reagents. Then proceed to assay the samples.

F. Extraction of NAD^+ from natural human skin:

1. Place a 5-mm biopsy of the natural human skin sample into a centrifuge tube containing 2.0 mL of $HClO_4$ and cap the centrifuge tube. Repeat the process for each 5-mm biopsy collected for an NAD^+ assay. Refrigerate the samples for 30 min. Transfer the samples to the -70 C freezer until further analysis.

2. Transfer the frozen natural human samples and the container of KOH/K_2HPO_4 to a sterile hood. Thaw the natural human skin samples. Determine the volume of KOH/K_2HPO_4 needed to titrate natural human skin samples Titration is determined in Section D.2.c. Add KOH/K_2HPO_4 to the samples, and this final volume is called the "total sample volume." Replace the lids on the centrifuge tubes and vortex the samples. Place the natural human skin samples in the refrigerator for a minimum of 15 min.

3. The samples are then centrifuged at 1,200 x g (2,500 rpm) for 10 min.

G. Preparation of "Buffer Volume" Solution:

1. Calculate the amount of "buffer volume" solution required for all NAD^+ samples. This is calculated by multiplying the total number of samples by 35 μL. Prepare an additional 3 mL more of "buffer volume" solution than is actually required. "Buffer volume" solution is prepared by titrating 0.5 M $HClO_4$ with KOH/K_2HPO_4, as determined in section D.2.c. After adding KOH/K_2HPO_4 to the $HClO_4$, vortex and place the solution into the refrigerator for a minimum of 15 min. Then centrifuge the "buffer volume" solution at 1,200 x g (2,500 rpm) for 10 min.

H. Analysis of NAD^+:

1. Thaw a set of NAD^+ standards, add 250 μL of the KOH/K_2HPO_4 solution, vortex, then incubate in the refrigerator for 15 min. Centrifuge the standards for 10 min at 1,200 x g (2.500 rpm)/

2. Add 75 μL of background (0 nM NAD^+ standard) and NAD^+ standards to each of four wells. This yields standards with NAD^+ levels approximately 750, 500, 250, 125, 62.5, 31.25, and 0 nM for standards described in Sections E.5.c. to E.5.h., respectively. Assay the background and standards on a plate separate from the samples. When the samples are assayed, transfer and combine 40 μL of sample (the "partial sample volume") with 35 μL of assay buffer (the "buffer volume") to a new 96-well microtiter plate for a "total diluent volume" of 75 μL. Include 75 μL of "buffer volume" with samples from background analysis. Record sample positions on Attachment #1.

3. Add 75 μL of Buffer II to each sample well under dimmed lights.

4. Add 20 µL of ADH (150 U/mL) to each sample well under dimmed lights to initiate the reaction. Cover the 96-well plate with a lid and place into the incubator for 15 min.

5. After 15 min, promptly add 20 µL of sodium iodoacetate (120 mM) solution. (Be sure to wipe the bottom of the plate thoroughly with lens paper to remove any condensation.

6. Immediately measure the absorbance of the wells at 540 nm using a Titertek Multiskan MCC/340 ELISA plate reader following Battelle SOP MREF V-002.

I. Calculation of NAD^+:

1. Regression analysis is performed on the standards to obtain slope (m) and y-intercept (b) values. Nanomolar concentrations of NAD^+ standards are the independent (x) variable, and background-subtracted absorbance values at 540 nm are the dependent (y) variable.

2. Background subtracted absorbance values at 540 nm for samples are converted to picomoles of NAD^+ per sample using the following series of calculations:

(a) Line equation $x = (y - b)/m$

where: m = slope
b = y-intercept
y = background-subtracted absorbance at 540 nm
x = $[NAD_+]$ (nM)

(b) Total NAD^+ (pmoles) = $[NAD^+]$ (nM) * Total diluent volume (µL) *

$$\frac{\text{Total sample volume (µL)}}{\text{partial sample volume(µL)}} * \frac{1000 \text{ pmoles}}{\text{nmole}} * \frac{1\text{L}}{10^6 \mu\text{L}}$$

J. References:

1. Mol, M.A.E., A.B.C. Van De Ruit, and A.W. Kluivers, NAD^+ Levels and Glucose Uptake of Cultured Human Epidermal Cells Exposed to Sulfur Mustard, Tox. App. Pharmacol. 98:159-165 (1989).

2. Meier, H. L., C.L. Gross, and B. Papirmeister, 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard) Decreases NAD^+ Levels in Human Leukocytes, Tox. Lett. 39:109-122 (1987).

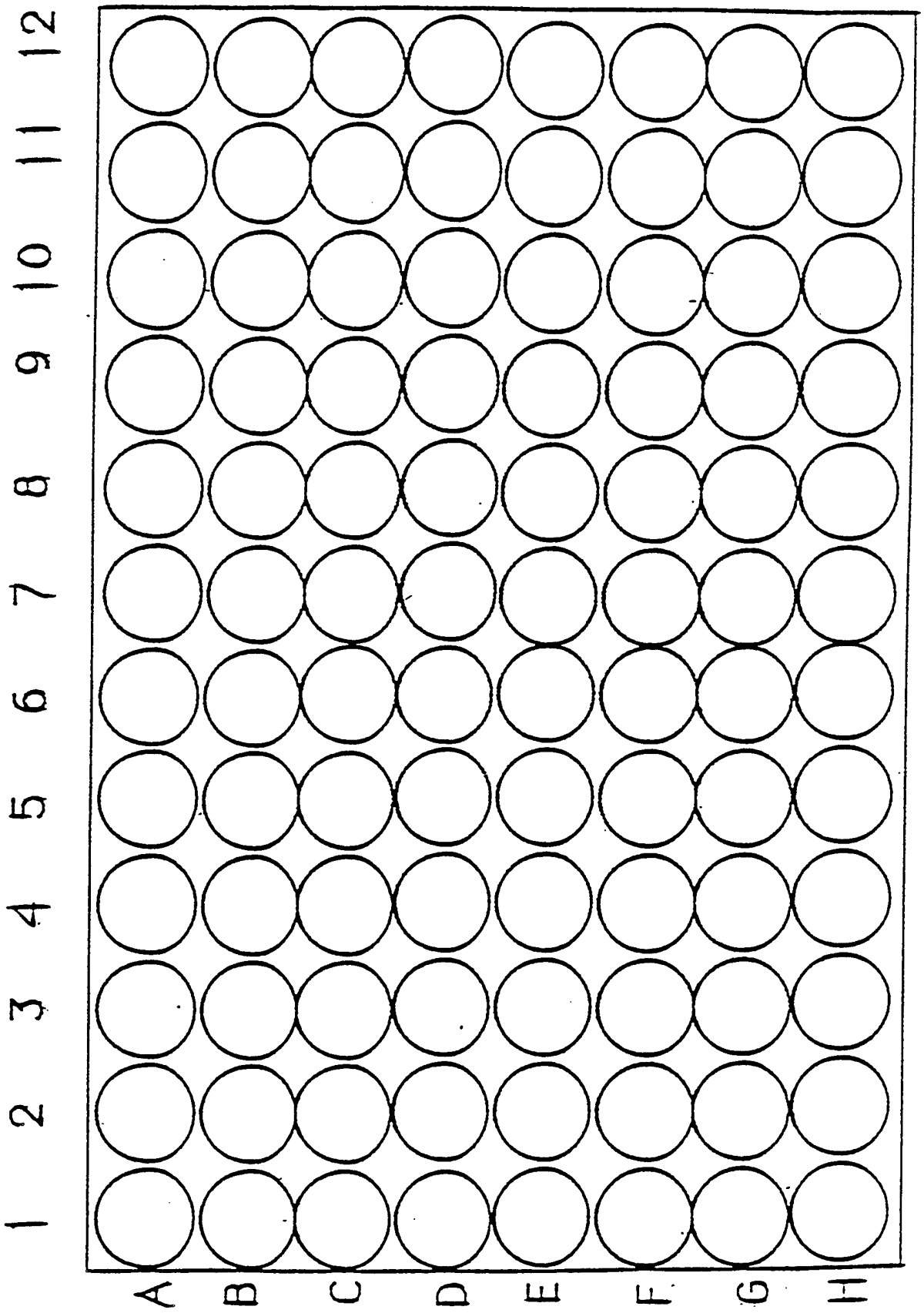
3. Jacobson, E.L. and M.K. Jacobson, Pyridine Nucleotide Levels as a Function of Growth in Normal and Transformed 3T3 Cells, Arch. Biochem. Biophys. 175:627-634 (1976).

4. Blank, J., Method for the Determination of Nicotinamide Adenine Dincleotide (NAD^+) Using the Multiskan MCC 340 MK II Elisa Plate Reader, Method #1/In Vitro-01. (1993)

Originated by: Christopher B. Logel, B.A. — Date: 4/11/94
Life Science Technician

Reviewed by: Thomas, H. Snider, B.S. — Date: 4/11/94
Research Scientist

ATTACHMENT #1



ATTACHMENT 5

MREF Method No. 30/In Vitro

METHOD FOR THE DETERMINATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) IN MatTek Epiderm™ USING THE MULTISKAN MCC 340 MK II ELISA PLATE READER

A. Statement of Work: This method describes the procedures used to measure nicotinamide adenine dinucleotide (NAD^+) levels in MatTek Epiderm™ samples. This procedure described in this method for NAD^+ analysis is based upon that of Jacobson and Jacobson (Section H.3.) adapted for use in a 96-well microtiter plate system.

B. Abbreviations and Reagents:

1. ADH - Alcohol Dehydrogenase (Sigma #A3263; 280 U/mg solid)
2. BSA - Bovine Serum Albumin
3. EDTA - Ethylenediamine tetraacetic acid
4. EtOH - Absolute ethanol
5. HCl - Hydrochloric Acid
6. $HClO_4$ - Perchloric Acid
7. K_2HPO_4 - dibasic Potassium Phosphate
8. KOH - Potassium Hydroxide
9. MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
10. NAD^+ - Nicotinamide Adenine Dinucleotide (oxidized)
11. PES - Phenazine ethosulfate
12. Bicine - N, N-Bis(2-hydroxyethyl)glycine

C. Precautions:

1. $HClO_4$ - $HClO_4$ is a potent oxidizing and caustic agent. Its use in the presence of organic solvents must be avoided. Dilutions of stock $HClO_4$ (60 - 70 percent $HClO_4$ solution) must be made in the biological safety cabinet as should all other work with diluted $HClO_4$ solution.

2. MTT and PES - These are labeled as being suspect carcinogens. They should be treated as such and weighed on a scale located in a vented cabinet. These are also light sensitive chemicals and should be weighed under reduced lighting. The container in which they are solubilized should be enclosed in foil.

D. Preparation of Reagents:

1. $HClO_4$ Dilution - All dilutions and additions of $HClO_4$ are to be performed in a biological safety cabinet that does not contain any organic solvents. The procedure requires two individuals. $HClO_4$ can be obtained as an approximate 60 to 70 percent solution in water. The stock bottle is kept in a secondary container stored in a cabinet that is void of any organic acids or solvents. The molarity of the stock $HClO_4$ solution is calculated as follows:

a. $$\frac{(1.768 \text{ gm/mL}) \times (1{,}000 \text{ mL/L})}{100.42 \text{ gm/mol}} = X \text{mol/L}$$

b. X multiplied by the percent of the $HClO_4$ solution and divided by 100 gives the molarity of the stock $HClO_4$ solution.

Prepare a 3 M $HClO_4$ solution in the biological safety cabinet over plastic backed paper using distilled water as the diluent. The solution is not used 60 days past the date of preparation.

c. One individual will remove the $HClO_4$ from the secondary container, open the cap of the $HClO_4$, and recap the bottle as soon as the aliquot is removed. The second individual removes and transfers the volume of stock $HClO_4$ to the Erlenmeyer flask. The $HClO_4$ is dispensed below the level of the distilled water. Just after dispensing, the serological pipette is filled with the diluted acid solution and this material is dispensed back into the Erlenmeyer flask. The Erlenmeyer is stoppered and clearly labeled with the following information: contents, molarity, date of preparation, and individual making the solution. The 3 M $HClO_4$ solution is kept refrigerated and will not be used 60 days past the date of preparation. Prepare 0.5 m $HClO_4$ by adding 30 mL of 3 M $HClO_4$ to 150 mL of distilled deionized water. The first individual replaces the $HClO_4$ in its secondary container and returns the secondary container to the acid cabinet.

2. Preparation of KOH/K_2HPO_4 Buffer:

a. Dissolve 28.74 gm of K_2HPO_4 in 400 mL of distilled water contained in a 500-mL beaker and adjust the pH to 7.8 with 3 N HCl.

b. Dissolve 28.05 gm of KOH in the phosphate buffered solution prepared above in Section 2.a., transfer the solution to a 500-mL volumetric flask, and fill to the 500 mL volume mark with distilled water. The solution contains 0.33 M K_2HPO_4 and 1 M KOH. The container will be labeled identifying the contents of the container, date of preparation, and the initials of the individual making the solution.

c. The KOH/K_2HPO_4 buffer is used to neutralize the $HClO_4$ solution. As slight variation in the $HClO_4$ and KOH/K_2HPO_4 solution may exist when the solutions are prepared, it is necessary to titrate the $HClO_4$ solution with KOH/K_2HPO_4 when either of the solutions is prepared. A 12 mL volume of 0.5 m $HClO_4$ is added to a 20 mL scintillation vial. A 5 mL volume of KOH/K_2HPO_4 buffer is added to the $HClO_4$, the solution mixed and the solution pH determined. Aliquots of 50 μL of KOH/K_2HPO_4 buffer is added to the scintillation vial until the resulting solution pH is between 6.8 and 7.0. The cumulative or total volume (mL) of KOH/K_2HPO_4 buffer

required to achieve this final pH (6.8 to 7.0) is computed. Dividing this value by 100 yields the volume in microliters of KOH/K_2HPO_4 required to neutralize 120 µL of 0.5 M $HClO_4$ solution. This volume should be 60 µL ± 5 µL. This volume, along with the date, is marked on the $HClO_4$ and KOH/K_2HPO_4 containers. If outside this range, either the $HClO_4$ or the KOH/K_2HPO_4 solutions should be prepared.

d. The solution is kept refrigerated and will not be used 60 days past the date of preparation.

3. Preparation of Assay Buffers:

a. Buffer I:

Reagent	Concentration	g/100 mL
Bicine	200 mM	3.264
EDTA	8.32 mM	0.310
BSA	1.66 mg/mL	0.166

Adjust to pH 7.8 with 1N KOH. Mark date of preparation, initial of individual preparing solution, and the expiration date which is 2 months when stored refrigerated.

b. Buffer II:

Reagent	Concentration	g/20 mL
MTT	0.84 mM	0.0068
PES	6.64 mM	0.0444
EtOH	500.00 mM	

(1) Prepare just prior to use. Dissolve the MTT and PES in 18.8 mL of Buffer I. Then add 1.2 mL of EtOH and mix. This is performed under reduced lighting.

(2) Wrap the container with aluminum foil immediately after preparation to protect from lighting. Discard solution after use.

4. Preparation of ADH: Prepare using distilled deionized water on the day of use and store refrigerated. Add 0.004 gm of ADH to 7.5 mL of diluent to yield a 150 U/mL solution. Discard solution after use.

5. Preparation of NAD^+ Standards:

a. 1.5×10^{-3} M Stock: Add 0.0054 gm of NAD^+ to 5 mL of 0.5 M $HClO_4$ and mix. Use 0.5 M $HClO_4$ as the diluent in subsequent dilutions.

b. 1.5×10^{-5} M (Working Stock): Add 0.05 mL of a. to 5 mL of diluent and mix.

c. 1.13×10^{-6} M: 0.75 mL of b. + 9.25 mL diluent.

d. 7.5×10^{-7} M: 4 mL of c. + 2 mL diluent.

e. 3.75×10^{-7} M: 3 mL of d. + 3 mL diluent.

f. 1.88×10^{-7} M: 3 mL of e. + 3 mL diluent.

g. 9.4×10^{-8} M: 3 mL of f. + 3 mL diluent.

h. 4.7×10^{-8} M: 3 mL of g. + 3 mL diluent.

i. Background: 0.5 M perchloric acid used for diluent.

Prepare the dilutions of the standards and place 0.5-mL aliquots into a series of Eppendorf centrifuge tubes. The tubes should be labeled indicating the standard letter, date of preparation, and individual preparing the dilution. The standards can be used for up to 1 month following preparation when stored frozen.

6. Preparation of Sodium Iodoacetate:

A 120 mM solution of sodium iodoacetate is prepared by adding 1.248 gm to 50 mL of distilled water. The solution container is marked to indicate contents, a 30-day expiration date, and the name of the individual preparing the solution.

E. Instrument Power Up:

1. Turn on the ELISA reader.

2. Press Measurement Mode - press arrow key until "single wavelength absorbance" appears on screen and press enter.

3. Using the numerical keys, press 540 and enter.

4. Press arrow key until "no background" appears on screen and press enter.

5. Insert the microtiter plate onto the reader platform and press start. The Titertek will print out the absorbance values. This printout will be appropriately identified, initialed, and dated. The printout must be photo copied for the study records, as the printout will fade with time.

6. Perform assays with the standards first to determine a standard curve, and to ensure that no problems exist with the reagents. Then proceed to assay the samples.

F. Extraction of NAD^+ from MatTek Epiderm™:

1. Place a 2-mm biopsy of the MatTek Epiderm™ sample into a centrifuge tube containing 0.3 mL of $HClO_4$ and cap the centrifuge tube. Repeat the process for each 2-mm biopsy collected for an NAD^+ assay. Refrigerate the samples for 30 min. Transfer the samples to the -70 C freezer until further analysis.

2. Transfer the frozen MatTek Epiderm™ samples and the container of KOH/K_2HPO_4 to a sterile hood. Thaw the MatTek Epiderm™ skin samples. Determine the volume of KOH/K_2HPO_4 needed to titrate MatTek Epiderm™ samples. Titration is determined in Section D.2.c. Add KOH/K_2HPO_4 to the samples, and this final volume is called "total sample volume". Replace the lids on the centrifuge tubes and vortex the samples. Place the MatTek Epiderm™ samples in the refrigerator for a minimum of 15 min.

3. The samples are then centrifuged at 1,200 x g (2,500 rpm) for 10 min.

G. Preparation of "Buffer Volume" Solution:

1. Calculate the amount of "buffer volume" solution required for all NAD^+ samples. This is calculated by multiplying the total number of samples by 35 µL. Prepare an additional 3 mL more of "buffer volume" solution than is actually required. "Buffer volume" solution is prepared by titrating 0.5 M $HClO_4$ with KOH/K_2HPO_4, as determined in section D.2.c. After adding KOH/K_2HPO_4 to the $HClO_4$, vortex and place the solution into the refrigerator for a minimum of 15 min. Then centrifuge the "buffer volume" solution at 1,200 x g (2,500 rpm) for 10 min.

H. Analysis of NAD^+:

1. Thaw a set of NAD^+ standards, add 250 µL of the KOH/K_2HPO_4 solution, vortex, then incubate in the refrigerator for 15 min. Centrifuge the standards for 10 min at 1,200 x g (2,500 rpm).

2. Add 75 µL of background (0 nM NAD^+ standard) and NAD^+ standards to each of four wells. This yields standards with NAD^+ levels approximately 750, 500, 250, 125, 62.5, 31.25, and 0 nM for standards described in Sections E.5.c. to E.5.h., respectively. Assay the background and standards on a plate separate from the samples. When the samples are assayed, transfer and combine 40 µL of sample (the "partial sample volume") with 35 µL of assay buffer (the "buffer volume") to a new 96-well microtiter plate for a "total diluent volume" of 75 µL. Include 75 µL of "buffer volume" with samples for background analyses. Record sample positions on Attachment #1.

3. Add 75 µL of Buffer II to each sample well under dimmed lights.

4. Add 20 µL of ADH (150 U/mL) to each sample well under dimmed lights to initiate the reaction. Cover the 96-well plate with a lid and place into the incubator for 15 min.

5. After 15 min, promptly add 20 µL of sodium iodoacetate (120 mM) solution. (Be sure to wipe the bottom of the plate thoroughly with lens paper to remove any condensation.

6. Immediately measure the absorbance of the wells at 540 nm using a Titertek Multiskan MCC/340 ELISA plate reader following Battelle SOP MREF V-002.

I. Calculation of NAD^+:

1. Regression analysis is performed on the standards to obtain slope (m) and y-intercept (b) values. Nanomolar concentrations of NAD^+ standards are the independent (x) variable, and background-subtracted absorbance values at 540 nm are the dependent (y) variable.

2. Background subtracted absorbance values at 540 nm for samples are converted to picomoles of NAD^+ per sample using the following series of calculations:

(a) Line equation $x = (y - b)/m$

where: m = slope
b = y-intercept
y = background-subtracted absorbance at 540 nm
x = $[NAD^+]$ (nM)

(b) Total NAD^+ (pmoles) = $[NAD^+]$ (nM) * Total diluent volume (µL) *

$$\frac{\text{Total Sample volume (µL)}}{\text{partial sample volume(µL)}} * \frac{\text{1000 pmoles}}{\text{nmole}} * \frac{\text{1L}}{10^6 \mu\text{L}}$$

J. References:

1. Mol, M.A.E., A.B.C. Van De Ruit, and A.W. Kluivers, NAD^+ Levels and Glucose Uptake of Cultured Human Epidermal Cells Exposed to Sulfur Mustard, Tox. App. Pharmacol. 98:159-165 (1989).

2. Meier, H. L., C.L. Gross, and B. Papirmeister, 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard) Decreases NAD^+ Levels in Human Leukocytes, Tox. Lett. 39:109-122 (1987).

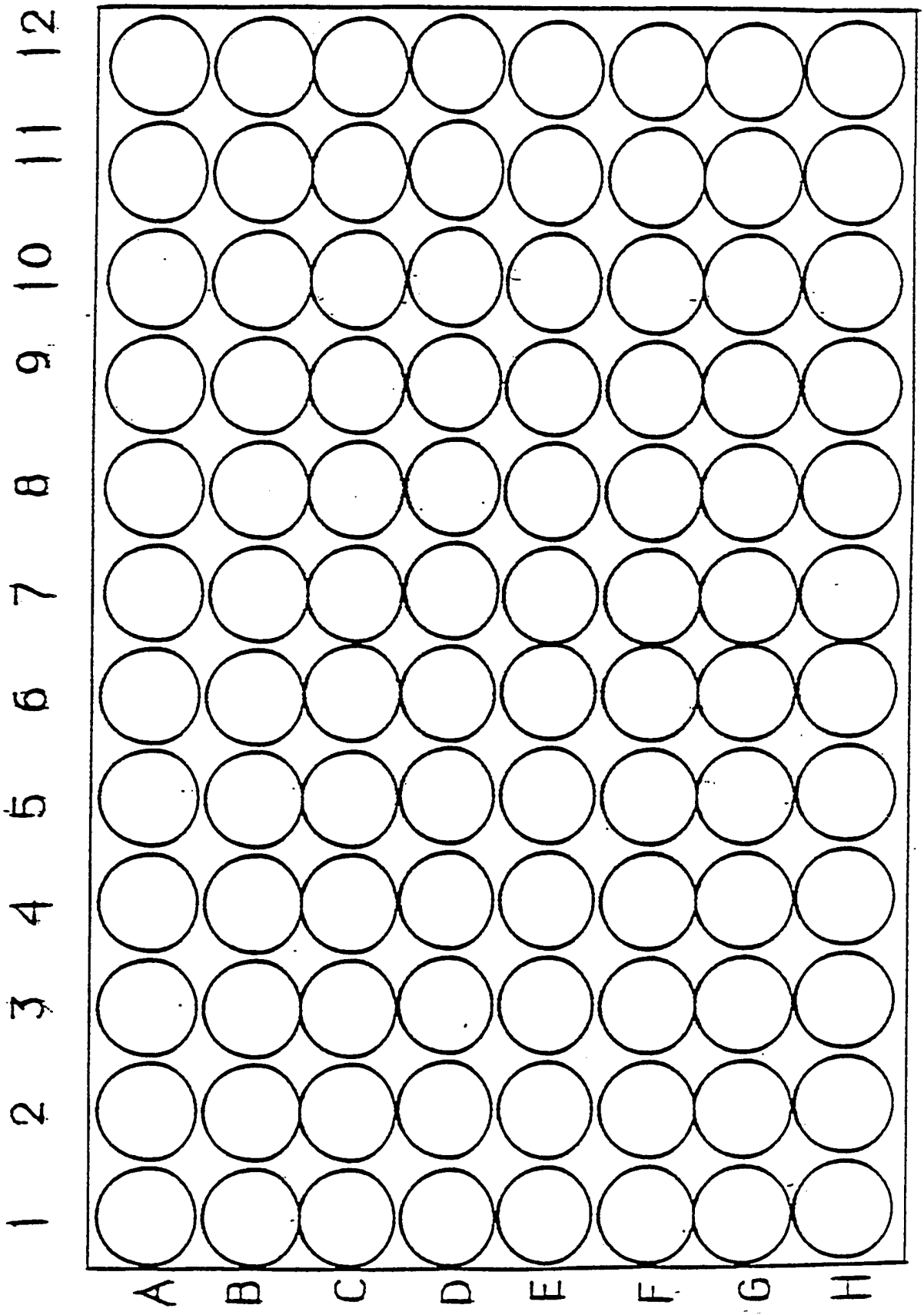
3. Jacobson, E.L. and M.K. Jacobson, Pyridine Nucleotide Levels as a Function of Growth in Normal and Transformed 3T3 Cells, Arch. Biochem. Biophys. 175:627-634 (1976).

4. Blank, J., Method for the Determination of Nicotinamide Adenine Dincleotide (NAD^+) Using the Multiskan MCC 340 MK II Elisa Plate Reader, Method #1/In Vitro-01. (1993)

Originated by: Christopher B. Logel, B.A.
Life Science Technician
4/11/94
Date

Reviewed by: Thomas, H. Snider, B.S.
Research Scientist
4/11/94
Date

ATTACHMENT #1



ATTACHMENT 6

MREF Method No. 25/In Vitro

METHOD FOR THE MEASUREMENT OF MatTek EpiDerm™ VIABILITY USING THE MTT PROCEDURE

A. Statement of Work: This method describes the application of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye uptake and hydrolysis procedure for determining the viability of MatTek EpiDerm™ sections.

B. Equipment:

Biological Safety Cabinet, 2-mm biopsy punch, lab coat, scale, forceps, carbon dioxide incubator, centrifuge, centrifuge tubes, aluminum foil, polystyrene flasks, rotator platform, ELISA plate reader, black permanent marker, Eppendorf pipettes, pipette tips, serological pipettes, pipette bulbs, pipette-aid, sterile petri dish, sterile 96-well microtiter plates, sterile 24-well tissue culture plates, sterile six-well tissue culture plates and latex gloves.

C. Materials:

MTT, Assay Medium, and isopropanol.

D. Abbreviations:

MatTek Corporation Epi-100 EpiDerm™ Human Skin Model System - A kit produced by MatTek Corporation that contains one, 24-well plate with 24 EpiDerm™ skin model samples (24, 10-mm round tissues per plate); 50 mL bottle of Assay Medium; 100 mL bottle of PBS Rinse Solution; 10 mL bottle of 1 percent Triton X-100 solution; four, six-well plates (sterile); two, 24-well plates (sterile); 24-well template, and Epiderm Data Sheet.

MatTek EpiDerm™ sample - An artificial living, human skin tissue section comprised of normal, human-derived epidermal keratinocytes (NEHK). The keratinocytes are cultured on a permeable cell culture MILLICELL insert.

MatTek Assay Medium - Assay medium supplied by MatTek Corporation for use with EPI-100 EpiDerm™ Human Skin Model System.

MatTek Maintenance Medium - Complex growth medium supplied by MatTek for use with EPI-100 EpiDerm™ Human Skin Model System.

PBS - Phosphate Buffered Saline.

MTT Extraction Solution - Isopropanol.

E. Precautions:

MTT is labeled as being a suspect carcinogen. It should be treated as such and weighed on a scale located in a vented cabinet. MTT is also a light-sensitive chemical and should be weighed under reduced lighting. The container in which it is solubilized should be enclosed in foil.

F. Experimental Design:

MTT dye solution is added to MatTek EpiDerm™ samples. After the specified incubation time, the tissue is rinsed, the hydrolyzed MTT (blue formazan product precipitated in mitochondria) is extracted, and its concentration determined spectrophotometrically.

G. Procedures:

1. Unpacking the MatTek Corporation Epi-100 EpiDerm™ Human Skin Model System:

   a. Unpack the MatTek EpiDerm™ samples from the shipping carton and note any damage to the shipping carton on Attachment #1. Keep tissues in shipping compartment or in the refrigerator until transferred to their six well maintenance plates.

   b. Record all lot numbers on the data sheets (Attachment #1).

   c. Under sterile conditions, transfer 35 mL of Maintenance Medium into a sterile test tube or flask. Cap the container and warm it to 37 C in an incubator for no longer than 1 hr.

   d. Place remaining growth and assay media in the refrigerator.

   e. In a sterile exhaust hood, unwrap four sterile six-well plates. Dispense 0.9 mL of pre-warmed Maintenance Medium into each well.

2. Transferring EpiDerm™ samples to Maintenance Plates:

   a. After donning a laboratory coat and clean pair of gloves, transfer the sealed MatTek EpiDerm™ sample shipping tray to the sterile hood. Unwrap the 24-well plate containing the MatTek EpiDerm™ samples. Remove the peel-off Lot Number/Production Date Label from the cover of the shipping tray and affix it to the data sheet (Attachment #1).

   b. Aseptically dispense 10 mL of Maintenance Medium into a sterile petri plate.

c. Remove the lid from the MatTek EpiDerm™ sample shipping plate. Record observations such as the color of agar and the condition of tissues at this time.

d. Grasp the side wall of the MatTek EpiDerm™ sample with sterile forceps and remove it from the agarose well. Gently rinse the bottom of the MatTek EpiDerm™ sample in the petri plate filled with pre-warmed maintenance medium to remove any adherent agarose. Place the MatTek EpiDerm™ sample into the center of a well of the six well maintenance plate.

Note: When transferring MatTek EpiDerm™ samples, be careful not to touch the surface of the tray except on the sides.

e. Repeat this process until all tissues have been transferred to a respective well and plate.

f. Upon completion of transfer, cover the six-well plate with a lid and label the lid to identify lot number, date and initials. Incubate the plates at 37 C, 5 percent carbon dioxide and 90 percent relative humidity until use. Wait 1 hr after the start of the incubation before initiating any assays.

g. Dispose of the shipping tray, lid, and rinsing medium in the biohazard waste receptacle.

h. Aspirate Maintenance Medium and replace it with fresh, pre-warmed Maintenance Medium every 24 hr.

3. Preparation of MTT Solution:

a. Calculate the amount of MTT Solution required for the MTT procedure (0.3 mL per well for whole samples or 1 mL per well for 2mm biopsy samples). Prepare slightly more solution than is actually required. MTT will be prepared to 2.0 mg/mL in Assay Medium.

b. For example, to prepare a 40 mL solution, add 80.0 mg of MTT powder to 40 mL of Assay Medium and mixed thoroughly.

c. Centrifuge the MTT Solution at 1,500 x g or 3,000 rpm for 5 min.

d. Carefully decant the supernate into a foil covered beaker or container. Cap the container and warm the solution to 37 C.

4. MTT procedure for whole EpiDerm™ sample:

(Table #1 can be used as a reference for each step of MTT procedure.)

a. Remove the six-well test plate(s) containing the MatTek EpiDerm™ sample sections from the incubator and place them into the sterile Biological Safety Cabinet.

b. Obtain a 24-well assay plate and label the lid with permanent marker to designate sample position for each sample used. Label a second 24-well plate in an identical manner for later use in the extraction step. Sample position can also be labeled on Attachment #2. Dispense 0.3 mL of MTT Solution (2.0 mg/mL; prewarmed to 37 C) into each sample well of the 24-well plate. One at a time, place each MatTek EpiDerm™ sample directly into the MTT solution in its designated well. It is necessary to include a Blank sample position for background analysis. Cover the plate with the lid and incubate the plate for 3 hr at 37 C, 5 percent carbon dioxide and 90 percent humidity on a rotating platform.

c. At the end of the 3 hr incubation, remove each MatTek EpiDerm™ sample individually and gently rinse with PBS to remove any residual MTT solution. Remove excess PBS by gently shaking the MatTek EpiDerm™ sample and then blotting the bottom with a Kimwipe. Place the inserts into the pre-labeled 24-well extraction plate.

d. Slowly dispense 2 mL of Extraction Solvent (isopropanol) per well, completely covering the MatTek EpiDerm™ sample. Cover the 24-well plate with parafilm and lid, then agitate samples on a rotating platform or similar type device for 2 hours at room temperature.

e. Using the table shown in Attachment #3, designate the wells of a 96-well plate as to what group or sample it will contain when the MTT extract solution from each well in the 24-well test plate is transferred to the 96-well reading plate.

f. At the end of 2 hr, vigorously reflux the solvent with a pipet against the MatTek EpiDerm™ sample to facilitate extraction and solubilization of the formazan product. Decant the liquid within each insert back into the well from which it was taken (i.e. mix the solution with the extractant in the well). Discard the insert.

g. Pipet the extractant solution up and down at least three times to ensure that the extraction solutions are well mixed. Pipet 200 µL of the extractant solution into the 96-well plate. Of the same extractant solution also place 100 µL into another well of the 96-well plate. Add 100 µL of fresh extraction solution to each of the 100 µL spent blue solvent wells in the 96-well plate (i.e., 1:1 dilution).

h. Read the 96-well plate on a microplate reader set at 570 nm. Use the absorbance from the Blank sample (isopropanol) as the sample background absorbance value.

i. Proceed to Calculation section.

5. MTT procedure for MatTek EpiDerm™ 2-mm biopsy samples:

(Table #1 can be used as a reference for each step of MTT procedure.)

a. Place plexiglass cutting block, 2 mm biopsy punch, forceps, 24-well assay plate and six well test plate containing the MatTek EpiDerm™ sample sections into the sterile Biological Safety Cabinet.

b. On the lid of the 24-well plate designate with permanent marker the sample position for each 2-mm biopsy sample assayed. This can also be recorded on Attachment #2. Dispense 1 mL of MTT Solution (2.0 mg/mL; prewarmed to 37 C) into each well that will be used of the 24-well test plate. Cover plate with lid.

c. Using the forceps, remove one MatTek EpiDerm™ sample from its well and place it on the cutting block. Punch a 2-mm biopsy from the MatTek EpiDerm™ sample. Grasp the edge of the biopsy with the forceps and transfer it into the MTT solution in its designated well. Repeat this process to obtain desired amount of samples. It is necessary to include a well for the background analysis. Incubate the plate for 2 hr at 37 C, 5 percent carbon dioxide and 90 percent humidity on a rotating platform.

d. At the end of the 2 hr incubation, remove the MTT solution by aspiration (one plate at a time) in the same sample position order previously designated.

e. Dispense 1 mL of Phosphate Buffered Saline (PBS) into each well (one plate at a time) and allow the plate to set at room temperature for 2 min. Aspirate the PBS solution in the same order in which it was added. Repeat this wash step and aspirate wash solution again.

f. Slowly dispense 125 µL of Extraction Solvent (isopropanol) per well. Cover six-well plate with parafilm and a lid, then agitate samples on a rotating platform or similar type device for 1 hr at room temperature.

g. At the end of 1 hr, vigorously reflux the solvent with a pipet against the MatTek EpiDerm™ sample biopsy mesh to facilitate extraction and solubilization of the formazan product. Using the table shown in Attachment #3, designate the wells of a 96-well

plate as to what sample it will contain when a 80 µL aliquot of the MTT extract solution from each well in the 24-well test plate is transferred to the 96-well reading plate.

h. Read the 96-well plate on a microplate reader set at 540 nm. Use the absorbance from the background well as the sample background absorbance value.

i. Proceed to Calculation section.

6. Calculation: The absorbance of the isopropanol background well will serve as the background absorbance value for all other wells. The background value is subtracted from all sample values prior to calculating the percent of control response. The effect of test chemical is calculated as follows:

$$\text{Percent Control} = \frac{OD_{\text{test chemical}}}{OD_{\text{vehicle}}} \times 100$$

Originated by: Christopher B. Logel, B.A. — Life Science Technician — 4/4/94 Date

Reviewed by: Thomas H. Snider, B.S. — Research Scientist — 4/4/94 Date

TABLE # 1

MODEL SYSTEM	MatTek Epiderm™	
Sample Size	2-mm Biopsy	Whole
MTT concentration: Amount of MTT per well: Plate size:	2 mg/mL 1 mL 24-well	2 mg/mL 300 µL 24-well
Incubation Time: 37 C 5 percent CO_2 ≥ 90 percent humidity rotation platform	2 hr	3 hr
Wash amount: (PBS Wash Solution) Wash time: (Tissue is washed twice)	1 mL 2 min	Remove insert, gently wash, then place in new 24-well plate with extraction solution.
Extraction amount: Extraction solution: Extraction time: - rotation platform - parafilm plate, cover with lid	125 µL Isopropanol 1 hr	2 mL Isopropanol 2 hr
Sample analysis amount: (96 well plate)	80 µL	1. 200 µL 2. 100 µL Sample + 100 µL Isopropanol
Absorbance:	540 nm	570 nm

ATTACHMENT # 1

MatTek EpiDerm™ DATA SHEET

MatTek EpiDerm™ Lot #:_____

Date Received:_____

Assay Medium Lot #:_____

Maintenance Medium Lot #:_____

Assay Types:_____

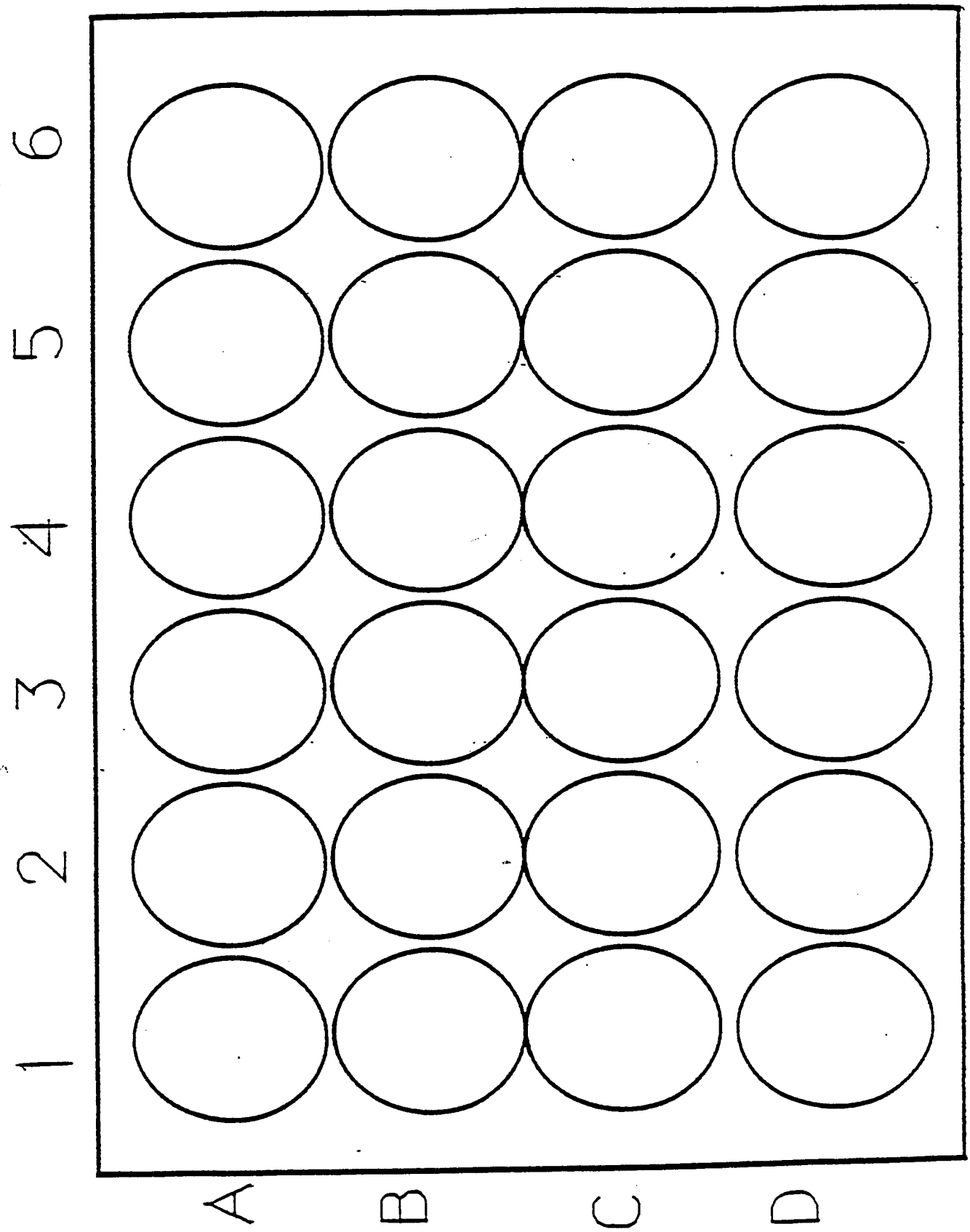
Dates of Assay:_____

Test Agents:_____

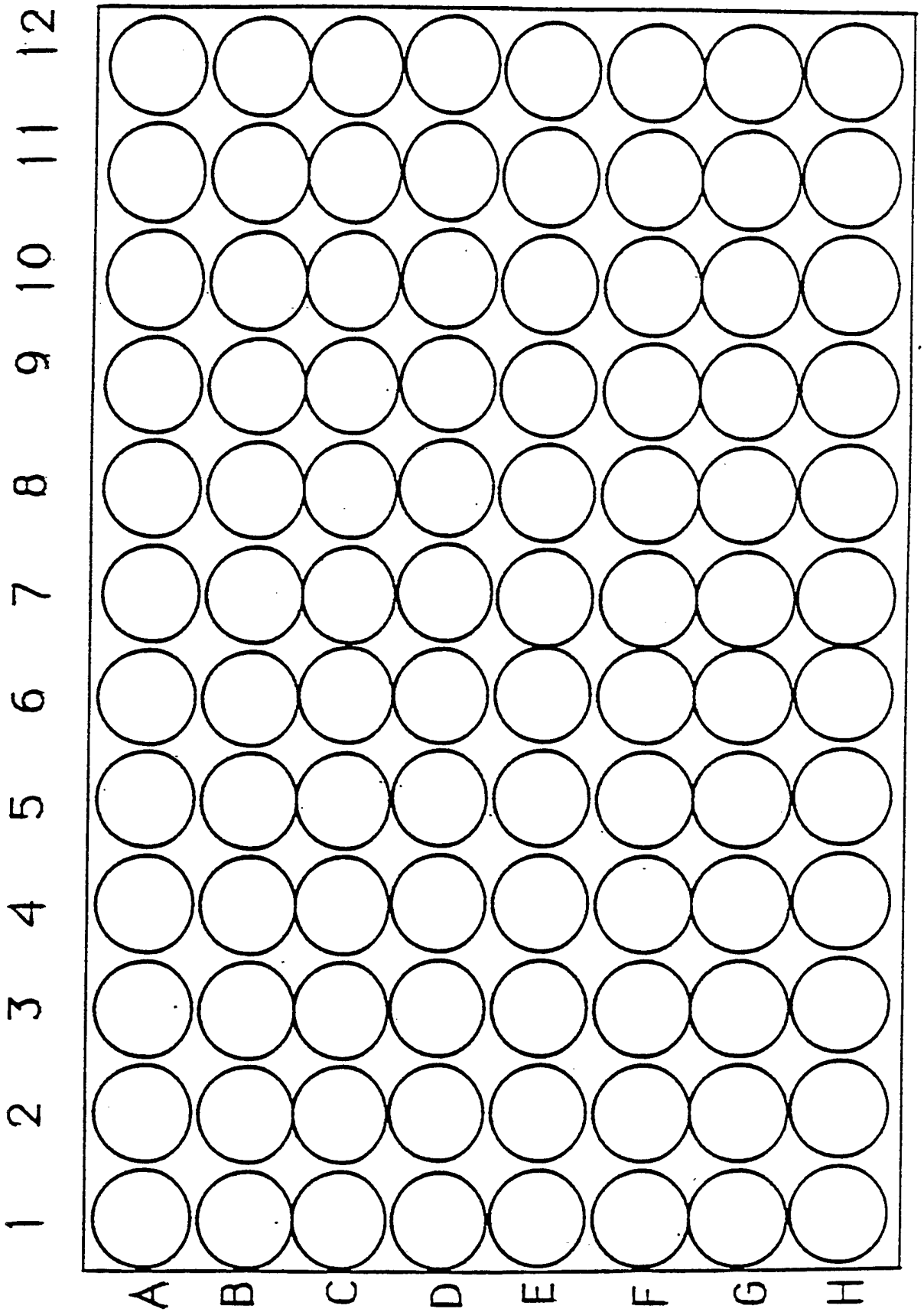
Technicians:_____

Comments:

ATTACHMENT # 2



ATTACHMENT # 3



ATTACHMENT 7

MREF Method No. 26/In Vitro

METHOD FOR THE MEASUREMENT OF ADVANCED TISSUE SCIENCES (ATS) SKIN^{2TM} VIABILITY USING THE MTT PROCEDURE

A. Statement of Work: This method describes the application of the MTT dye uptake and hydrolysis procedure for determining the viability of ATS skin^{2TM} sections.

B. Equipment:

Biological Safety Cabinet, 5-mm biopsy punch, lab coat, scale, forceps, carbon dioxide incubator, centrifuge, centrifuge tubes, aluminum foil, polystyrene flasks, rotator platform, ELISA plate reader, black permanent marker, Eppendorf pipettes, pipette tips, serological pipettes, pipette bulbs, pipette-aid, sterile 96-well microtiter plates, sterile 24-well tissue culture plates, sterile six-well tissue culture plates and latex gloves.

C. Materials:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Assay Medium, and isopropanol.

D. Abbreviations:

ATS skin^{2TM}ZK1300 Kit - A kit produced by ATS that contains one ATS skin^{2TM} tray (24, 11 x 11 mm tissues per tray); one-125 mL bottle of Maintenance Medium; one-250 mL bottle of Serum-Free Assay Medium; 1 vial containing two Blank Meshes; four, six-well assay plates; and four, six-well plates with MILLICELLS.

ATS skin^{2TM} - An living, three-dimensional human skin tissue that has dermal, epidermal and corneal layers. Each section is composed of a fibroblast dermal layer and a multi-layered keratinocyte epidermis and stratum corneum.

ATS Maintenance Medium - A complex growth media supplied with ATS skin^{2TM}ZK1300 Kit that contains Dulbecco's Modified Eagle's Medium (DMEM) and 5 percent Fetal Bovine Serum (FBS) with supplements.

ATS Serum-Free Assay Medium - An assay media supplied with ATS skin^{2TM}ZK1300 Kit that contains Dulbecco's Modified Eagle's Medium with supplements.

PBS - Phosphate Buffered Saline.

MTT Extraction Solution - Isopropanol.

procedure with one adjacent corner. Be careful not to touch the surface of the tray except on the sides. With the one end of the tray opened, continue to peel the lid back upon itself. When nearly all the rows of wells have been exposed, then the lid towards the one remaining corner.

c. Use sterile forceps to remove the ATS skin^{2TM} sections, one at a time, from the agarose matrix. Take care to minimize the amount of tissue surface that comes in contact with the forceps during this process. Also be careful not to pick up any agarose.

d. Place the ATS skin^{2TM}, dermis-side down (i.e., the side in contact with the agarose), into the center of the MILLICELL. Repeat this process until all tissues have been transferred.

e. Upon completion of transfer, cover the the six-well plate with the lid, label the lid to identify lot number, date and initials. Incubate the plates at 37 C, 5 percent carbon dioxide and 90 percent relative humidity until use.

f. Dispose of the shipping tray, and lid in the biohazard waste receptacle.

g. Aspirate Maintenance Medium and replace it with fresh, pre-warmed Maintenance Medium every 24 hr.

3. Preparation of MTT Solution:

a. Calculate the amount of MTT Solution required for MTT procedure (2 mL per well for whole samples or 1 mL per well for 5mm biopsy samples). Prepare an additional 3 mL more of solution than is actually required. MTT will be prepared to 2.0 mg/mL in Assay Medium. For example to prepare 40 mL of solution, 80.0 mg of MTT powder is added to 40 mL of Assay Medium and mixed thoroughly in a foil enclosed centrifuge tube.

c. Centrifuge the MTT Solution at 1,500 x g or 3,000 rpm for 5 min.

d. Carefully decant the supernate into a clean, foil covered beaker or container. Label the container appropriately. Cap the container and warm the solution to 37 degree C.

4. MTT procedure for whole ATS skin^{2TM} samples:

   (Table #1 can be used as a reference for each step of the MTT procedure.)

   a. Remove the six-well test plate containing the ATS skin^{2TM} sections from the incubator and place them into the sterile Biological Safety Cabinet.

   b. Obtain a new six-well assay plate and label the lid with permanent marker to designate sample position for each sample used. Sample position can also be labeled on Attachment #1. Dispense 2 mL of MTT Solution (2.0 mg/mL; prewarmed to 37 C) into each sample well of the new six-well test plate. One at a time, submerge each ATS skin^{2TM} directly into the MTT solution in its designated well. It is necessary to include a Blank Mesh sample for the background analysis. Incubate the plate for 2 hr at 37 C, 5 percent carbon dioxide and 90 percent humidity on a rotating platform.

   c. At the end of the 2 hr incubation, aspirate the MTT solution (one plate at a time) in the same sample position order previously designated.

   d. Dispense 1 mL of Phosphate Buffered Saline (PBS) into each well (one plate at a time) and allow the plate to set at room temperature for 2 min. Aspirate the PBS solution in the same order in which it was added. Repeat this wash step and aspirate wash solution again.

   e. Slowly dispense 4 mL of Extraction Solvent (isopropanol) per well. Cover the six-well plate with parafilm and lid, then agitate samples on a rotating platform or similar type device for 1 hr at room temperature.

   f. At the end of 1 hr, vigorously reflux the solvent with a pipet against the ATS skin^{2TM} mesh to facilitate extraction and solubilization of the formazan product. Using the table shown in Attachment #2, designate the wells of a 96-well plate as to what group or sample it will contain when a 100 μL aliquot of the MTT extract solution from each well in the six-well test plate is transferred to the 96 well reading plate. Add 100 μL of fresh extraction solution to each of spent blue solvent wells in the 96-well plate (i.e., 1:1 dilution).

   g. Read the 96-well plate on a microplate reader set at 540 nm. Use the absorbance from the Blank Mesh sample as the sample background absorbance value.

   h. Proceed to Calculation section.

5. MTT procedure for ATS skin^{2TM} 5mm biopsy samples:

(Table #1 can be used as a reference for each step of MTT procedure.)

a. Place plexiglass cutting block, 5-mm biopsy punch, forceps, 24-well assay plate and six-well test plate containing the ATS skin^{2TM} sections into the sterile Biological Safety Cabinet.

b. On the lid of the 24-well plate designate with permanent marker the sample position for each 5-mm biopsy sample assayed. This can also be recorded on Attachment #3. Dispense 1 mL of MTT Solution (2.0 mg/mL; prewarmed to 37 C) into each well that will be used of the new 24-well test plate. Cover the plate with a lid.

c. Using the forceps, remove one ATS skin^{2TM} from its MILLICELL and place it on the cutting block. Punch a 5-mm biopsy from one of the corners of the sample. (It is possible to obtain 4, 5 mm biopsy samples, from each ATS skin^{2TM}.) Gently grasp the edge of the biopsy with the forceps and transfer it into the MTT solution in its designated well. Repeat this process to obtain the desired number of biopsy samples. It is necessary to include a well for the background (blank mesh) analysis. Incubate the plate for 2 hours at 37 degree C, 5 percent carbon dioxide and 90 percent humidity on a rotating platform.

d. At the end of the 2 hr incubation, aspirate the MTT solution (one plate at a time) in the same sample position order previously designated.

e. Dispense 1 mL of Phosphate Buffered Saline (PBS) into each well (one plate at a time) and allow the plate to set at room temperature for 2 min. Aspirate the PBS solution in the same order in which it was added. Repeat this wash step and aspirate wash solution again.

f. Slowly dispense 1 mL of Extraction Solvent (isopropanol) per well. Cover the 24-well plate with parafilm and a lid, then agitate the samples on a rotating platform or similar type device for 1 hr at room temperature.

g. At the end of 1 hr, vigorously reflux the solvent with a pipet against the ATS skin^{2TM} biopsy mesh to facilitate extraction and solubilization of the formazan product. Using the table shown in Attachment #2, designate the wells of a 96-well plate as to what group or sample it will contain when a 100 μL aliquot of the MTT extract solution from each well in the 24-well test plate is transferred to the 96-well reading plate.

h. Read the 96-well plate on a microplate reader set at 540 nm. Use the absorbance from the background (blank mesh) well as the sample background absorbance value.

i. Proceed to Calculation section.

6. Calculation: The absorbance from the Blank Mesh sample or background well serves as the background absorbance value for all other wells. The background value is subtracted from all sample values prior to calculating the percent of control response. The effect of test chemical is calculated as follows:

$$\text{Percent Control} = \frac{OD_{\text{test chemical}}}{OD_{\text{vehicle}}} \times 100$$

Originated by: Christopher B. Logel 4/4/94
Christopher B. Logel, B.A. Date
Life Science Technician

Reviewed by: Thomas H. Snider 4/4/94
Thomas H. Snider, B.S. Date
Research Scientist

TABLE #1

MODEL SYSTEM	ATS skin™2 Model ZK1300	
Sample Size	5-mm Biopsy	Whole
MTT concentration: Amount of MTT per well: Plate size:	2 mg/mL 1 mL 24-well	2 mg/mL 2 mL six well
Incubation Time: 37 C 5% percent CO_2 ≥ 90 percent humidity rotation platform	2 hr	2 hr
Wash volume: (PBS Wash Solution) Wash time: (Tissue is washed twice)	1 mL 2 min	1 mL 2 min
Extraction volume: Extraction solution: Extraction time: - rotation platform - parafilm plate, cover with lid	1 mL Isopropanol 1 hr	4 mL Isopropanol 1 hr
Sample analysis amount: (96-well plate)	100 µL	100 µL Sample + 100 µL Isopropanol
Absorbance:	540 nm	540 nm

Skin^{2TM} DATA SHEET

ADVANCED TISSUE SCIENCES
TEL: (619) 450-5730
FAX: (619) 450-5703
TOLL FREE: (800) 252-7273

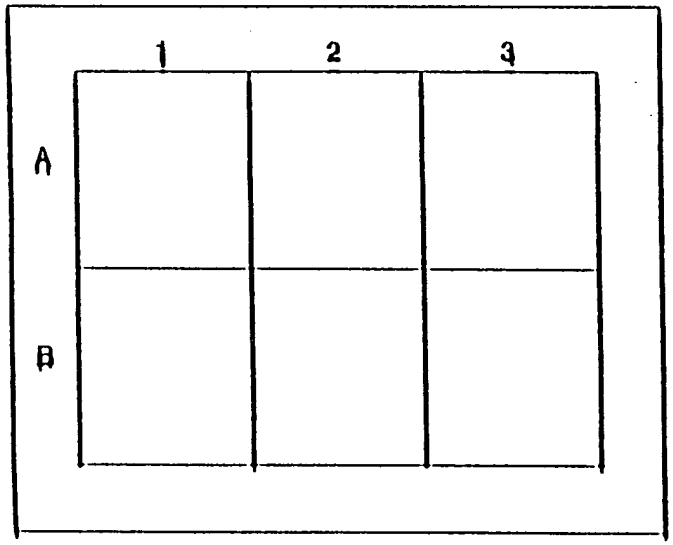
Skin² Kit Part # _____ Date Received _____ Blank Mesh Lot # _____

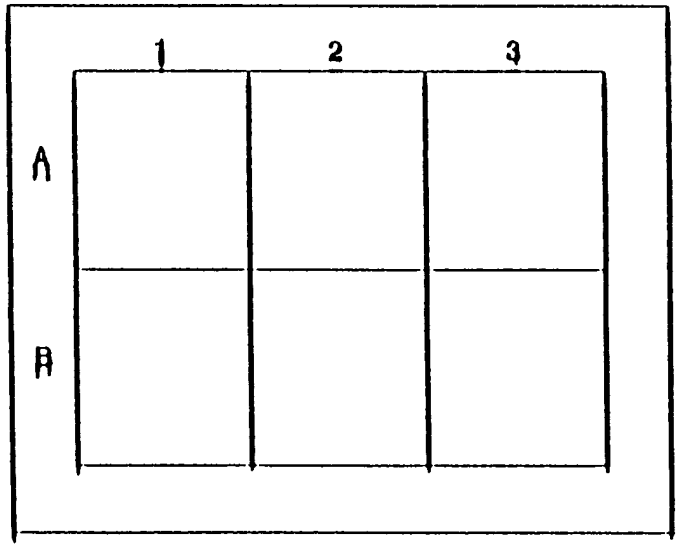
Skin² Kit Lot # _____ Date(s) of Assay _____ Assay Medium Lot # _____

Assay Type(s) _____ Date(s) of Assay _____ Maintenance Medium Lot # _____

Technician(s) _____ Concentrations _____ Skin² Substrate Lot # _____

Test Agents _____

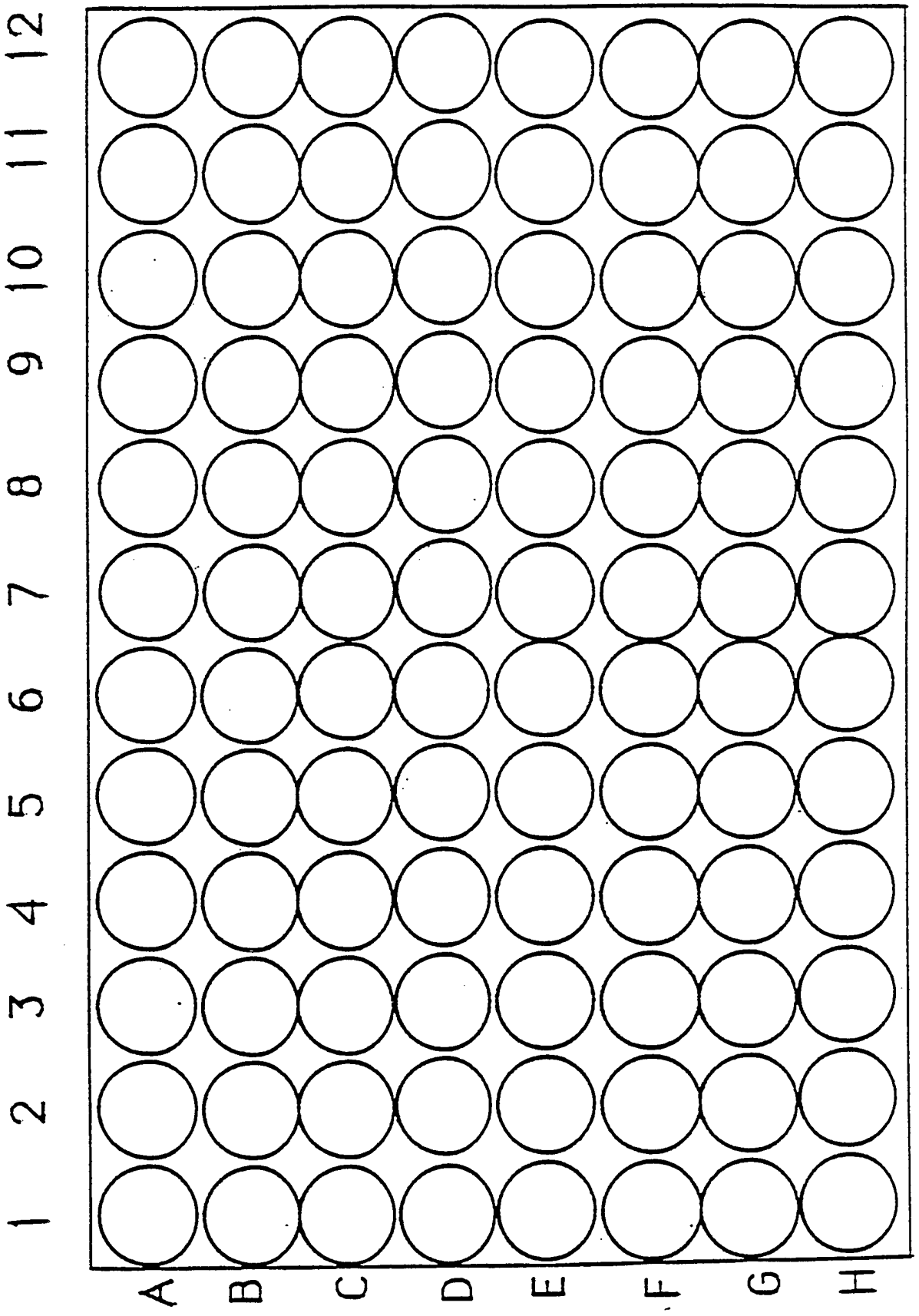




ATTACHMENT #1

10933 North Torrey Pines Road
La Jolla, CA 92037

ATTACHMENT #2



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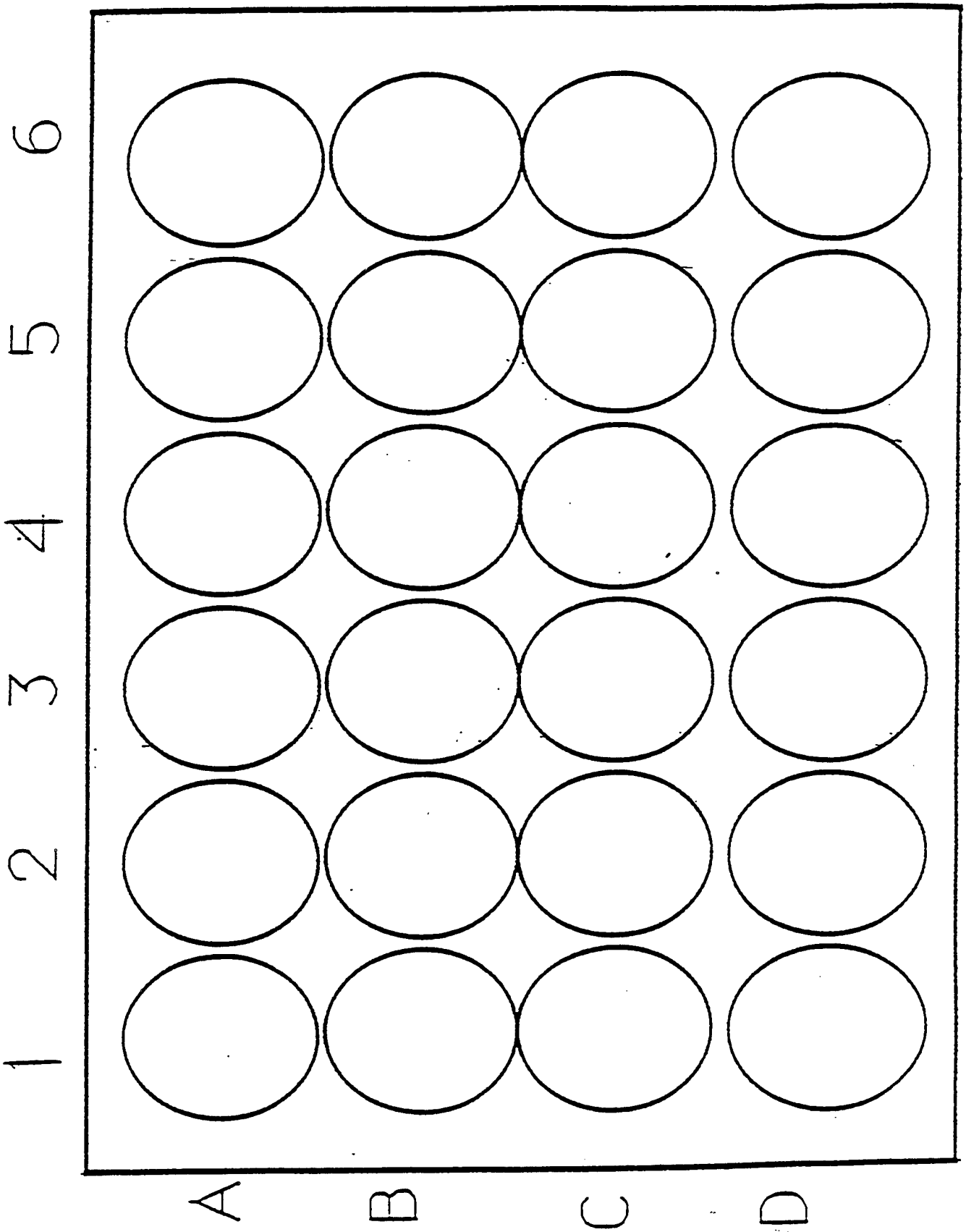


ATTACHMENT 8

MREF Method No. 27/In Vitro

METHOD FOR THE MEASUREMENT OF NATURAL HUMAN SKIN VIABILITY USING THE MTT PROCEDURE

A. Statement of Work: This method describes the application of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye uptake and hydrolysis procedure for determining the viability of natural human skin obtained through the Cooperative Human Tissue Network (CHTN).

B. Equipment:

Biological Safety Cabinet, 5-mm biopsy punch, dermatome, dermatome blades, lab coat, scale, forceps, carbon dioxide incubator, refrigerator (4 degree C), centrifuge, centrifuge tubes, aluminum foil, polystyrene flasks, rotator platform, ELISA plate reader, black permanent marker, Eppendorf pipettes, pipette tips, serological pipettes, pipette bulbs, pipette-aid, sterile petri dish, 96 well microtiter plates, 24 well tissue culture plates and latex gloves.

C. Materials:

MTT, Advanced Tissue Sciences (ATS) ATS Maintenance Medium - A complex growth media supplied by ATS that contains Dulbecco's Modified Eagle's Medium (DMEM) and 5 percent Fetal Bovine Serum (FBS) with supplements. Maintenance Medium, and UNISOL™.

D. Abbreviations:

PBS - Phosphate Buffered Saline.

MTT Extraction Solution - UNISOL™.

E. Precautions:

MTT is labeled as being a suspect carcinogen. It should be treated as such and weighed on a scale located in a vented cabinet. MTT is also a light-sensitive chemical and should be weighed under reduced lighting. The container in which it is solubilized should be enclosed in foil.

UNISOL™ - A tissue solubilizer prepared by ISOLAB Incorporated. UNISOL™ is a very caustic, corrosive chemical solution. Wash skin at once if contacted.

F. Experimental Design:

MTT dye solution is added to natural human skin for 2 hr later, the MTT hydrolyzed (blue formazan product precipitation mitochondria) is extracted and its concentration is determined spectrophotometrically.

G. Procedures:

1. Receiving the natural human skin:

a. Natural human skin is received through the CHTN, which is locally operated through The Ohio State University.

b. For each sample received, record any pertinent information, such as I.D. number, date, etc., on datasheet Attachment #1. Also record on Attachment #1 any observations such as the color of the skin and the condition of the tissues.

c. Set the dermatome to a cutting thickness of 1 mm. Place the dermatome and the dermatome board into the sterile hood.

d. Record maintenance medium type (ATS or KGM®) and lot number on Attachment #1.

e. Under sterile conditions, transfer 20 mL of Maintenance Medium into a sterile 50 mL centrifuge tube. This tube will be used to maintain the natural human skin.

f. Fill the bottom of a sterile petri dish with maintenance media.

g. After donning a laboratory coat and clean pair of gloves, transfer the container of human skin to a sterile hood. Remove the skin section(s) from the container with sterile forceps and place the skin into the sterile petri dish filled with maintenance media (enough to keep the skin moist). Take care to minimize the amount of tissue surface that comes in contact with the forceps during this process. Spread the tissue out flat and then transfer it to the dermatome board.

Note: Keep the skin moist with maintenance media at all times.

h. Dermatome the skin and then lay it out flat on a sterile gauze. Wrap the skin up in the gauze and then place it into the pre-filled maintenance media centrifuge tube. Pour out excess maintenance medium (only use enough to saturate the gauze). Store the skin in the refrigerator (4 C) until use.

i. Dispose of the skin shipping container and other items that were in contact with the skin in the biohazard waste receptacle.

j. Aspirate Maintenance Medium and replace it with fresh Maintenance Medium every 24 hours.

3. Preparation of MTT Solution:

a. Calculate the amount of MTT Solution required for MTT procedure (1 mL per well for 5-mm biopsy samples). Prepare an additional 3 mL of solution than is actually required. MTT will be prepared to 2.0 mg/mL in Assay Medium. For example, to prepare 40 mL of solution, 80.0 mg of MTT powder is added to 40 mL of Assay Medium and mixed thoroughly in a foil enclosed centrifuge tube.

c. Centrifuge the MTT Solution at 1,500 x g or 3,000 rpm for 5 min.

d. Carefully decant the supernate into a clean, foil covered beaker or container. Label the container. Cap the container and warm the solution to 37 C.

4. MTT procedure for natural human skin 5-mm biopsy samples.

(Table #1 can be used as a reference for each step of MTT procedure.)

a. Place plexiglass cutting block, a 5-mm biopsy punch, forceps, 24-well assay plate and natural human skin into the sterile Biological Safety Cabinet.

b. On the lid of the 24-well plate, designate with permanent marker the sample position for each 5-mm biopsy sample assayed. This can also be recorded on Attachment #2. Dispense 1 mL of MTT Solution (2.0 mg/mL; prewarmed to 37 C) into each well that will be used of the 24-well test plate. Cover the plate with a lid.

c. Using the forceps, remove the natural human skin from the centrifuge tube and place it on the cutting block. Punch a 5-mm biopsy from the natural human skin. Gently grasp the edge of the biopsy with the forceps and transfer it into the MTT solution in its designated well. Repeat this process to obtain desired amount of samples. It is necessary to include a well for the background analysis. Incubate the plate for 2 hr at 37 C, 5 percent carbon dioxide and 90 percent humidity on a rotating platform.

d. At the end of the 2 hr incubation, aspirate the MTT solution (one plate at a time) in the same sample position order previously designated.

e. Dispense 1 mL of Phosphate Buffered Saline (PBS) into each well (one plate at a time) and allow the plate to set at room temperature for 2 min. Aspirate the PBS solution in the same order in which it was added. Repeat this wash step and aspirate wash solution again.

f. Slowly dispense 1 mL of UNISOL™ per well. Cover the 24-well plate with parafilm and a lid, then agitate the samples on a

rotating platform or similar type device overnight at room temperature.

g. The next day, using the table shown in Attachment #3, designate the wells of a 96-well plate as to what group or sample it will contain when MTT extract solution from each well in the 24-well test plate is transferred to the 96-well reading plate.

h. Pipet the extractant solutions up and down several times to ensure that they are well mixed. Pipet 100 µL of the extractant solution into the 96-well plate. Of the same extractant solution, place 50 µL into another well of the 96 well plate. Dilute all spent blue 50 µL samples with an additional 50 µL of fresh Unisol (i.e., 1:1 dilution).

h. Read the 96-well plate on a microplate reader set at 540 nm. Use the absorbance from the Blank Sample Well (Unisol) as the sample background absorbance value.

6. Calculation: The absorbance from the Blank Mesh sample or background well serves as the background absorbance value for all other wells. The background value is subtracted from all sample values prior to calculating the percent of control response. The effect of test chemical is calculated as follows:

$$\text{Percent Control} = \frac{OD_{\text{test chemical}}}{OD_{\text{vehicle}}} \times 100$$

Originated by: Christopher B. Logel 4/4/94
Christopher B. Logel, B.A. Date
Life Science Technician

Reviewed by: Thomas H. Snider 4/4/94
Thomas H. Snider, B.S. Date
Research Scientist

TABLE # 1

MODEL SYSTEM	HUMAN SKIN
Sample Size	5-mm Biopsy
MTT concentration: Amount of MTT per well: Plate size:	2 mg/mL 1 mL 24-well
Incubation Time: 37 C 5h percent CO_2 ≥ 90 percent humidity rotation platform	2 hr
Wash amount: (PBS Wash Solution) Wash time: (Tissue is washed twice)	1 mL 2 min
Extraction amount: Extraction solution: Extraction time: - rotation platform - parafilm plate, cover with lid	1 mL Unisol Overnight
Sample analysis amount: (96-well plate)	1. 100 µL sample 2. 50 µL sample + 50 µL Unisol
Absorbance:	540 nm

ATTACHMENT # 1

HUMAN NATURAL SKIN DATA SHEET

Natural Human Skin ID#:_____

Date Received:_____

Assay Medium Lot #:_____

Maintenance Medium Lot #:_____

Assay Types:_____

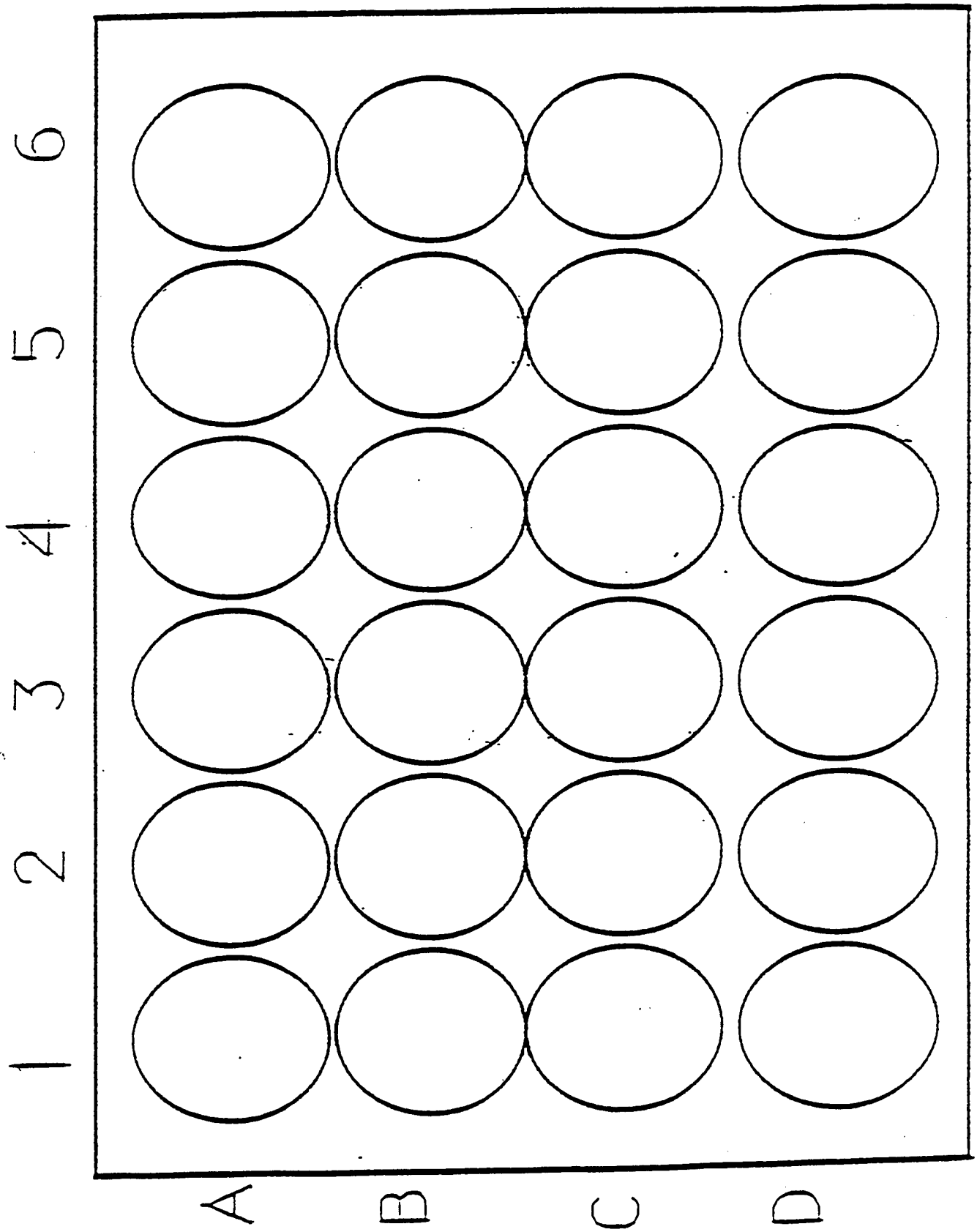
Dates of Assay:_____

Test Agents:_____

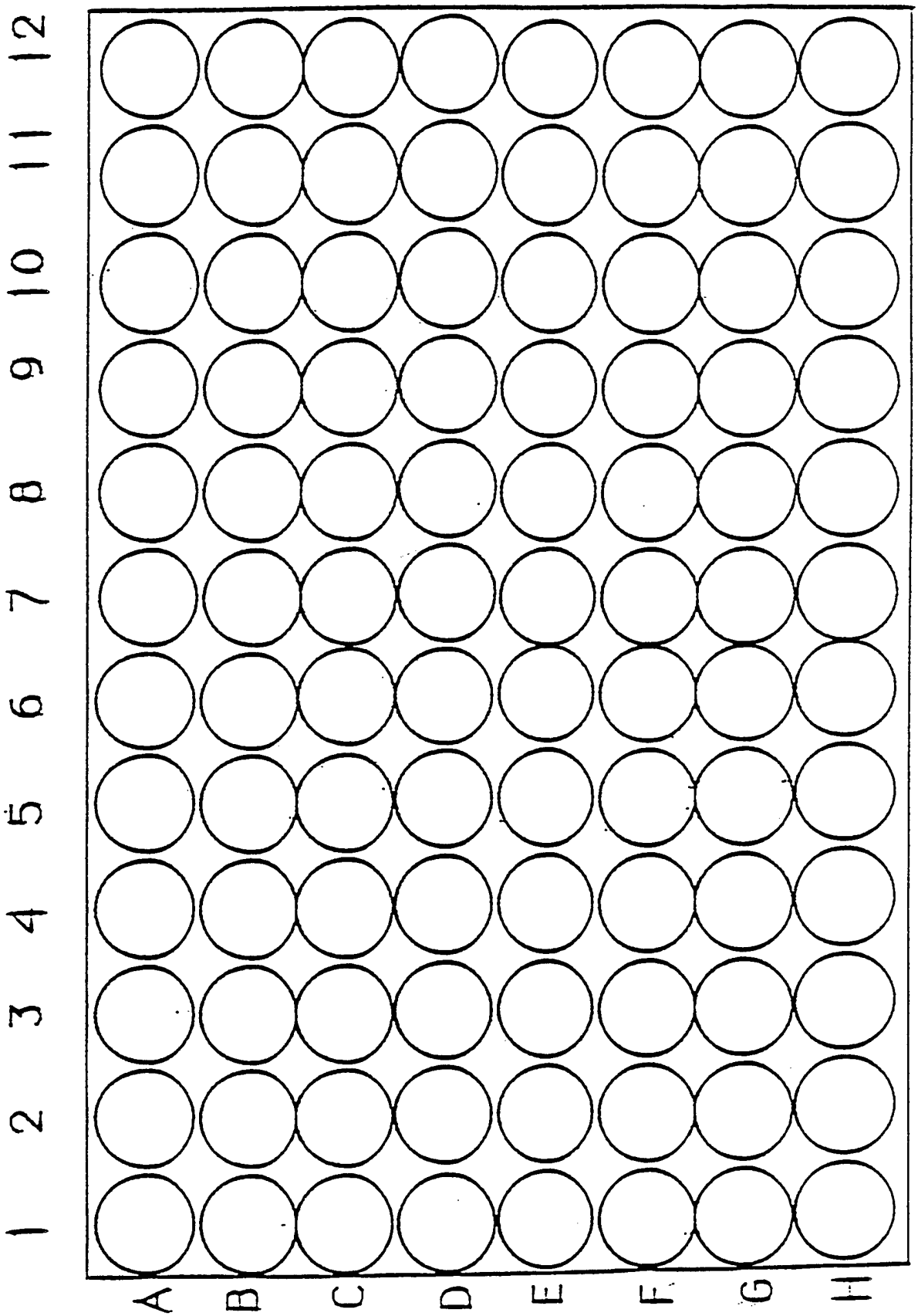
Technicians:_____

Comments:

ATTACHMENT # 2



ATTACHMENT # 3



ATTACHMENT 9

Dr. Pellegrini's Light Microscopy Report on Advanced Tissue Science Human Skin Equivalent and Natural Human Skin Tissues

THE OHIO STATE UNIVERSITY HOSPITALS

University Hospitals

410 West 10th Avenue
Columbus, OH 43210-1228

DATE: FEBRUARY 15, 1994

TO: TOM SNIDER

FROM: ARTHUR E. PELLEGRINI, M.D.

SUBJECT: SUMMARY STATEMENT

The human skin equivalent appeared to show little lot to lot variation. Human skin showed little deterioration over day 0 to day 7 except for increased perivascular space on day 5 and day 7.

The ATS sections showed greater variability than human skin but there did not appear to be definite lot to lot variability. Days 1, 2, and 3 show 1+ necrosis, 1+ sloughing and 1+ clefting when compared to day 0 specimens though occasionally 1+ changes were noted on day 0 specimens. The only specimens which showed 3 or less cells in thickness of the epidermal layer were day 0 specimens, suggestive proliferation of epidermal cells with time.

Respectfully,

Arthur E. Pellegrini, M.D.
Surgical Pathology

Degree of Necrosis

1+ Patchy single necrotic keratinocytes
2+ Focal confluent necrotic keratinocytes
3+ Broad areas of necrosis

Perinuclear Halo

1+ Patchy
2+ Focal confluent
3+ Broad

Sloughing (separation of epidermis from the support tissue)

1+ <25%
2+ 26-50%
3+ >51%

Clefting (separation between groups of cells in the epidermis)

1+ patchy < 25%
2+ focal confluent 26-50 %
3+ broad > 51%

Upper Dermal Vascular Spaces

1+ Mild
2+ Moderate
3+ Marked

Papillary Dermal Edema

1+ Mild
2+ Moderate
3+ Marked

Phase 1/Natural Human Skin
Battelle

Day	Lot/ID#	Sample	Epidermal		Necrosis			Perinuclear			Upper Dermal			Papillary		
		Number	Thickness					Halo			Vascular Spaces			Dermal Edema		
			0 - 4	>4	A	P	D	A	P	D	A	P	D	A	P	D
0	9310C253	68		X	X				X	1+	X			X		
0	9310C253	69		X	X				X	1+	X			X		
0	9310C253	70		X	X				X	3+	X			X		
0	9310C277	71		X	X				X	2+	X			X		
0	9310C277	72		X	X				X	3+	X			X		
0	9310C277	73		X	X				X	3+	X			X		
3	9310C277	74		X	X				X	3+	X			X		
3	9310C277	75		X	X				X	3+	X			X		
3	9310C277	76		X	X				X	1+	X			X		
5	9310C277	77		X	X				X	2+		X	1+	X		
5	9310C277	78		X	X				X	3+		X	1+	X		
5	9310C277	79		X	X				X	3+	X			X		
7	9310C277	80		X	X				X	3+		X	1+	X		
7	9310C277	81		X	X				X	2+		X	1+	X		
7	9310C277	82		X	X				X	3+		X	1+	X		
0	9311C053	83		X	X				X	3+	X			X		
0	9311C053	84		X	X				X	3+	X			X		
0	9311C053	85		X	X				X	2+	X			X		
0	9311C104R	107		X	X				X	1+	X			X		
0	9311C104R	108		X	X				X	1+	X			X		
0	9311C104R	109		X	X				X	2+	X			X		

A=Absent
P=Present
D=Degree (see tables)

Phase 1/Advanced Tissue Science Sections
Battelle

Day	Lot/ID#	Sample Number	Epidermal Thickness		Necrosis			Perinuclear Halo			Sloughing			Clefting		
			0-3	>3	A	P	D	A	P	D	A	P	D	A	P	D
0	*1472-091493-138K-20E*	65	X		X			X			X				X	1+
0	*1472-091493-138K-20E*	66		X	X			X			X			X		
0	*1478-091493-138K-20E*	67	X		X			X						X		
0	*1478-102693-138K-13E*	113		X	X			X				X	1+			
0	*1478-102693-138K-13E*	114		X		X	1+	X			X			X		
0	*1478-102693-138K-13E*	115	X		X			X			X				X	3+
0	*1481-111693-138K-15B*	119		X	X			X			X			X		
0	*1481-111693-138K-15B*	120		X	X			X			X			X		
0	*1481-111693-138K-15B*	121		X	X			X			X			X		
0	*1481-111693-138K-7D*	122		X		X	1+	X			X			X		
0	*1481-111693-138K-7D*	123	X			X	1+	X				X	1+		X	1+
0	*1481-111693-138K-7D*	124		X		X	1+	X				X	1+	X		
1	*1481-111693-138K-7D*	125		X		X	1+		X	1+		X	1+	X		
1	*1481-111693-138K-7D*	126		X		X	1+		X	1+	X				X	1+
1	*1481-111693-138K-7D*	127		X		X	1+		X	1+	X			X		
2	*1481-111693-138K-7D*	128		X		X	1+		X	1+		X	1+	X		
2	*1481-111693-138K-7D*	129		X		X	1+		X	1+		X	1+		X	1+
2	*1481-111693-138K-7D*	130		X		X	1+		X	1+		X	1+		X	1+
3	*1481-111693-138K-7D*	131		X		X	1+		X	2+	X			X		
3	*1481-111693-138K-7D*	132		X		X	1+		X	1+	X			X		
3	*1481-111693-138K-7D*	133		X		X	1+		X	1+		X	1+		X	1+

A = Absent
P = Present
D = Degree (see tables)

ATTACHMENT 10

Dr. Pellegrini's Light Microscopy Report on the First Set of Four Lots of MatTek Human Skin Equivalent Tissue

T · H · E
OHIO
STATE
UNIVERSITY
HOSPITALS

University Hospitals

410 West 10th Avenue
Columbus, OH 43210-1228

August 25, 1994

Thomas H. Snider
Battelle
Research Scientist
Medical Research and Evaluation Facility
JM-3
505 King Avenue
Columbus, OH 43201

Dear Tom:

Enclosed are the tabulated results of the MREF slides. The findings in these slides are:

1. Epidermal thickness decreases with time

2. The keratin layer thickens with time

3. The plastic embedded slides are readable

Sincerely,

Arthur E. Pellegrini, M.D.
Surgical Pathology

Phase 1/S-12
MREF
Battelle

Day		Sample Number	Epidermal Thickness		Necrosis			Reactive Nucleoli		Keratin Layer Vacuolization			Keratin Thickness
		LM #	0 - 6	>6	A	P		A	P	A	P		
0		44		X	X			X		X			< 0.025
0		45		X	X			X		X			fragmented
0		46		X	X			X		X			< 0.025
0		47		X	X			X		X			< 0.025
0		48		X	X			X		X			< 0.025
0		49		X	X			X		X			< 0.025
1		50	X		X			X		X			fragmented
1		51	X		X			X		X			fragmented
1		52	X		X			X		X			> 0.025
2		53	X		X			X		X			< 0.025
2		54	X		X			X		X			> 0.025
2		55	X		X			X		X			> 0.025
3		56	X		X			X		X			> 0.025
3		57	X		X			X		X			> 0.05
3		58	X		X			X		X			> 0.025
0		59		X	X			X		X			> 0.025
0		60		X	X			X		X			< 0.025
0		61		X	X			X		X			< 0.025
0		62		X	X			X		X			< 0.025
0		63		X	X			X		X			< 0.025
0		64		X	X			X		X			< 0.025

A = Absent
P = Present
D = Degree

10 lines = 0.025 mm
20 lines = 0.05 mm

ATTACHMENT 11

Dr. Pellegrini's Light Microscopy Report on the Second Set of Four Lots of MatTek Human Skin Equivalent Tissue

THE OHIO STATE UNIVERSITY
UNIVERSITY MEDICAL CENTER

University Hospitals
Division of Surgical Pathology

E 421 Doan Hall
410 West 10th Avenue
Columbus, OH 43210-1228
Phone (614) 293-5905
Fax (614) 293-4715

April 26, 1994

Thomas H. Snider
Battelle
Research Scientist
Medical Research and Evaluation Facility
505 King Avenue
Columbus, OH 43201

Dear Thomas:

Enclosed are the findings regarding the MATTEK specimens.

Sincerely,

Arthur E. Pellegrini, M.D.

Peter B. Baker, M.D. 293-3886
Susan J. Bona, M.D. 293-8496
Filiberto Cavazos, M.D. 293-5905
Kathryn P. Clausen, M.D. 292-4330
Julio C. Cruz, M.D. 293-3856
Kevin F. Forsthoefel, M.D. 293-8946
Arif Hameed, M.D. 292-2003
Sedi Keyhani-Rofagha, M.D. 293-3880
Joel G. Lucas, M.D. 293-4874
William L. Marsh, M.D. 293-5450
Robert V. O'Toole, M.D. 293-4880
Arthur E. Pellegrini, M.D. 293-4460

Summary of findings regarding Mattek Specimens

The parameters evaluated were epidermal thickness, keratinocyte dyskeratosis/necrosis, the presence of reactive nuclei characterized by nuclear enlargement and increase in the size of nucleoli, vacuolization in the keratin layer and thickness of the keratin layer.

Epidermal thickness was greater than six cells in day 0 specimens and day 1 specimens. Epidermal thickness was less than six cells in day 2 and day 3 specimens.

Confluent keratinocyte dyskeratosis/necrosis (3+) was seen in some day 0 specimens (lots 290 and 291). Other day 0 specimens showed absent or 1+ dyskeratosis/necrosis. Day 1 from lot 288 showed absent dyskeratosis/necrosis. Day 2 and day 3 specimens showed absent dyskeratosis/necrosis.

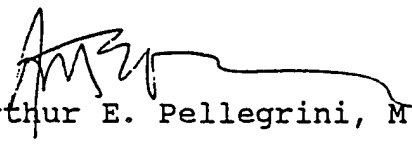
Reactive nuclei were present to some degree in specimens from all days though less in days 0 specimens.

Keratin layer vacuolization was most prominent in day 2 and day 3 specimens although some day 0 specimens showed a keratin layer ~~of~~ vacuolization.

The keratin layer thickened with time with most day 0 specimens being less than .025 mm in thickness in all day 3 specimens being greater than 0.05 mm in thickness.

In summary, the number of epidermal cells decreased with time, the keratin layer thickened with time and more vacuolization of the keratin layer was noted with time. Necrosis/dyskeratosis was most prominent on day 0 specimens probably reflecting changes occurring during transport and storage.

Respectfully submitted,



Arthur E. Pellegrini, M.D.

Battelle/Mattek Tables

Degree of Dyskeratosis/Necrosis

Absent
1+ Patchy single cells
2+ Many single cells
3+ Confluent
4+ Extensive

Keratin layer vacuolization

Absent
1+ Rare
2+ Focal
3+ Extensive

Phase 1/MatTek
Battelle

Day	Lot/ID#	Sample	Epidermal		Necrosis			Reactive		Keratin Layer			Keratin
		Number	Thickness					Nucleoli		Vacuolization			Thickness
			0 - 6	>6	A	P	D	A	P	A	P	D	
0	288	LM 134		X	X			X		X			> 0.025
0	288	LM 135		X	X			X		X			< 0.025
0	288	LM 136		X	X				X	X			> 0.025
1	288	LM 137		X	X			X		X			> 0.025
1	288	LM 138		X	X			X		X			> 0.025
1	288	LM 139		X	X				X	X			> 0.025
2	288	LM 140	X			X	1+		X		X	1+	> 0.025
2	288	LM 141	X			X	1+		X		X	1+	> 0.025
2	288	LM 142	X			X	1+		X		X	2+	> 0.025
3	288	LM 143	X			X	1+		X		X	2+	> 0.05
3	288	LM 144	X			X	1+		X		X	2+	> 0.05
3	288	LM 145	X			X	1+		X		X	2+	> 0.05
0	290	LM 146		X		X	3+		X	X			< 0.025
0	290	LM 147		X		X	3+		X	X			< 0.025
0	290	LM 148		X	X				X	X			< 0.025
0	291	LM 149		X		X	3+		X		X	1+	< 0.025
0	291	LM 150		X		X	3+		X	X			< 0.025
0	291	LM 151		X		X	4+	X			X	2+	< 0.025
0	424	LM 152		X		X	1+		X	X			< 0.025
0	424	LM 153		X		X	1+		X	X			< 0.025
0	424	LM 154		X		X	1+		X	X			< 0.025

A=Absent
P=Present
D=Degree (see tables)

10 lines = 0.025 mm
20 lines = 0.05 mm

ATTACHMENT 12

Dr. Monteiro-Riviere's Report with Photographs Characterizing
All Three Tissue Types by Transmission Electron Microscopy

AD_____

CONTRACT NO: DAMD17-89-C-9050
BATTELLE SUBCONTRACT NO: 53155-G155524

TITLE: Evaluation of human skin equivalents for studying HD- induced dermatotoxicology

PRINCIPAL INVESTIGATOR: Nancy A. Monteiro-Riviere, Ph.D.

CONTRACTING ORGANIZATION: North Carolina State University
College of Veterinary Medicine
Cutaneous Pharmacology and Toxicology Center
Raleigh, North Carolina 27606

REPORT DATE: January 31, 1994

TYPE OF REPORT: Interim Report on Phase I

PREPARED FOR: Battelle Columbus Operations
and
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21702-5012

FOREWORD

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Mary A Martinez Rios 1/31/94

PI - Signature DATE

EVALUATION OF HUMAN SKIN EQUIVALENTS AND NATURAL HUMAN SKIN FOR STUDYING HD-INDUCED DERMATOTOXICOLOGY

Human skin equivalents (HSEs) purchased from Advanced Tissue Science (ATS) and Mat Tek (MT) and natural human breast skin (NHS) obtained from a tissue bank were studied by transmission electron microscopy (TEM) for an intralot and an interlot study of morphological variability. Three sample areas were harvested from each of four lots (Day 0) to assess intralot and interlot variability. In addition, three sample areas from a Day 0 lot were stored in culture (3 days for HSEs and 7 days for NHS) to assess viability. Twenty-one sample areas were harvested from each tissue type for a total of 63 samples (Tables 1 and 2).

In the initial stage of this study, 24 Mat Tek samples (EM1 through EM24) were processed and embedded before a fixation problem at Battelle was discovered. In addition, the 19 initial ATS samples (EM25 through EM43) were examined by TEM and found to be necrotic, and therefore not included in this study. No additional tissue samples from ATS were assessed until this problem was corrected. All tissues processed for this study are summarized in Table 1. Table 2 lists all samples evaluated by TEM.

MATERIALS AND METHODS

Tissue samples were received, trimmed, and placed in vials containing cold (4°C) half strength Karnovsky's fixative by Battelle personnel. The samples were placed on wet ice and shipped overnight to the laboratory of Dr. Nancy Monteiro-Riviere. The tissue samples were then trimmed to the appropriate processing size (approximately $1mm^2$ for NHS and 2mm x 4mm for the HSEs), post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, cleared in acetone, infiltrated, and embedded in Spurr resin. Thick (1 μm) sections were cut using glass knives and stained with 1% toluidine blue. These sections were then screened with an Olympus BH-2 photomicroscope to assess the overall morphology of the sample and to

determine the representative area ($< 0.5mm^2$) for evaluation by TEM. Thin sections (800 Å) were cut with a diamond knife, picked up on 200/75 mesh copper grids, stained with uranyl acetate and Reynolds' lead citrate, and then viewed with a Philips EM410 transmission electron microscope operating at an accelerating voltage of 80KV. Each section was thoroughly evaluated and representative areas were photographed.

RESULTS

NATURAL HUMAN SKIN

Light microscopy: The epidermis of the NHS was made up of the keratinized cells of the stratum corneum, the flattened cells of the stratum granulosum, the polyhedral cells of the stratum spinosum, and the cuboidal to columnar cells of the stratum basale. The entire thickness of the viable epidermis (stratum granulosum, stratum spinosum, and stratum basale) varied from about 45-65μm and up to 83μm in the rete pegs. The compact stratum corneum measured from 6-11μm in thickness. Langerhans cells and their processes were frequently found within the epidermis. The Day 0 samples contained numerous melanocytes in the two sample lots of black skin (ID# 9310C253 and ID# 9310C277; Figure 1). The severity of intercellular epidermal edema varied from slight (ID# 9310C277) to moderate (ID# 9311C104R) or severe (ID# 9311C053; Figure 2).

Morphological differences were apparent in the skin stored in the culture medium (ID# 9310C277) prior to fixation. In all samples, the culture medium was retained above (Figure 2) and between the cell layers of the stratum corneum. A few vacuoles were present in the stratum spinosum cells of Day 3 samples, which increased in Day 5. At Day 7, additional vacuoles were present in the stratum spinosum and stratum basale cells. Dark basal pyknotic cells were also present within the epidermis. Samples within the same lot (intralot) were similar. The underlying dermis in all samples contained the normal cell types, including macrophages, fibroblasts, and mast cells.

Electron microscopy: The layers of the NHS stratum corneum (Day 0) consisted of keratinized stratified squamous cells connected by desmosomes. Membrane coating granules and desmosomes were fairly numerous within the upper stratum granulosum layer. The membrane coating granules in the upper stratum granulosum were occasionally seen fusing to the lower stratum corneum cell layer (Figure 3). The distribution of tonofilament bundles, keratohyalin granules, and lipids was normal. Occasional degenerating cells indicative of normal epidermal keratinization were seen in the stratum granulosum layer.

The polyhedral stratum spinosum cells and cuboidal stratum basale cells were connected to adjoining cells by desmosomes. Intercellular epidermal edema in the stratum spinosum and stratum basale layers ranged from slight (ID# 9310C277; Figure 4) to moderate (ID# 9311C104R; Figure 5) or severe (ID# 9311C053; Figure 6). Mitochondria within some samples occasionally contained swollen or ruptured cristae. Melanocytes were found within the stratum basale layer and stage IV melanosomes were scattered throughout the stratum spinosum and stratum basale layers of two lots (ID# 9310C253 and ID# 9310C277; Figure 7). In samples having severe intercellular epidermal edema, basal cells were flat and the nuclei contained no discrete nucleolus. Stellate tonofilament bundles were numerous within the cells, while Langerhans cells and their processes were scattered throughout the stratum spinosum layers. Langerhans cell granules were noted in some of the processes.

The basement membrane was highly convoluted and appeared normal (Figure 7). Hemidesmosomes were aligned along the plasma membrane of the basal cells. The electron lucent lamina lucida separating the basal cell plasma membrane from the underlying lamina densa contained thin anchoring filaments (Figure 8). Anchoring fibrils as well as microfibril bundles extended from the lamina densa into the dermis (Figure 8).

Slight cellular degeneration was apparent in the Day 3 samples. The residual culture medium detected by light microscopy appeared as an amorphous substance between the stratum corneum layers of all Day 3, Day 5, and Day 7 samples. Occasional necrotic cells and organelles were scattered throughout the stratum spinosum and stratum basale cell layers

(Figure 9) of Day 3 samples. Slight nuclear envelope separation and degenerative mitochondria with ruptured cristae were sometimes present in these layers (Figure 9). Langerhans cells usually contained the typical rod-shaped Langerhans granules, an indented nucleus, swollen and ruptured mitochondria and an electron lucent cytoplasm, but lack desmosomes and tonofilaments (Figure 10). Paranuclear clefts present in some stratum basale cells (Figure 11) sometimes contained membranous whorls.

At Day 5, the stratum corneum appeared more fragmented and more electron lucent. Membrane coating granules had degenerated to small vacuoles in the stratum granulosum. Cytoplasmic vacuoles were scattered throughout the stratum spinosum and stratum basale layers. Paranuclear clefts (Figure 12) were also present and more numerous than Day 3. Also, necrotic cells with chromatin clumping were obvious. The stratum basale became distorted, with a loss of cellular integrity. Focal areas of the convoluted basement membrane had thickened hemidesmosomes that appeared to extend into the lamina lucida.

Overall cellular degeneration evidenced by a decrease in cellular integrity was greater at Day 7. Mitochondria were damaged and cristae completely ruptured, leaving only cytoplasmic vacuoles throughout the cells. An increase in vacuoles, paranuclear clefts, and degenerative organelles throughout the epidermis was noted. Tonofilaments did not appear as recognizable bundles, but rather as homogeneous hyalin masses within these cells (Figure 13). In addition, vacuoles and ruptured mitochondria were found within the dermal fibroblasts.

MAT TEK

Light microscopy: The Mat Tek HSE (Epiderm™) is a model consisting of normal human derived epidermal keratinocytes which were cultured to form a multilayered differentiated model of human epidermis. This HSE consisted of a thick and compact stratum corneum layer, a prominent stratum granulosum layer, a stratum spinosum layer, a stratum basale layer, and a thin acellular dermis (Figure 14). This entire skin culture was supported on a microporous membrane approximately 28μm in thickness. The average stratum corneum thickness measured 19μm in Day 0 samples, 25μm in Day 1 samples, 27-56μm in Day 2 samples, and 47-64μm in Day 3 samples. The border between the stratum corneum and the stratum granulosum was very irregular, with numerous fingerlike projections of the stratum corneum layer extending into the stratum granulosum (Figure 15). The cells of the stratum granulosum varied in shape from circular to flattened with prominent keratohyalin granules. Although the stratum granulosum layer was typically 1-3 cells thick, focal areas up to 5 cell layers were sometimes noted. Cells of the stratum spinosum were polyhedral and contained circular nuclei. The stratum basale cell layer was made up of cuboidal to slightly elliptical cells containing circular to oval nuclei with compact nucleoli and cytoplasmic tonofilaments.

Although the thickness of the viable epidermis varied between 80-130μm in all samples, no rete pegs were present. Since the thickness of the stratum corneum increased from Day 0 to Day 3, the remaining layers of the epidermis subsequently decreased in thickness. The overall morphology of the epidermis appeared comparable to that of human skin, with the exception of focal areas of swollen stratum granulosum cells and basal intercellular edema (Lot# 312B; Figure 16) and moderate intercellular epidermal edema and vacuoles within the stratum basale cells (Lot# 312C; Figure 17). The underlying dermis in Day 0 to Day 3 samples varied in thickness from 3-8μm (Figure17).

The epidermal morphology at Day1, Day 2, and Day 3 was similar to that of Day 0. The notable exception was that projections of stratum corneum into the stratum granulosum

appeared progressively more pronounced. One MT sample (EM61, Lot# 312B) exhibited abnormal stratification of the epidermis (Figure 18).

Electron microscopy: The majority of the stratum corneum of Day 0 samples consisted of fairly compact cell layers connected with occasional desmosomes. Isolated areas of the stratum corneum were highly disorganized. Remnants of numerous degenerated cellular organelles such as membrane coating granules devoid of lipid as well as filaments were present intracellularly and intercellularly throughout the organized (Figure 19) and disorganized (Figure 20) layers. In some cases, the upper surface of the stratum corneum contained electron dense profiles and fibrous strands embedded in an amorphous ground substance (Figure 21). This may represent an area of undifferentiated keratin containing the remnants of membrane coating granules and mitochondria. The bottom layer of the stratum corneum varied a great deal in thickness and often sent fingerlike projections extending down between the cells of the stratum granulosum (Figure 22).

The large, flattened cells of the stratum granulosum contained rounded keratohyalin granules and were attached to adjacent cells by desmosomes (Figure 22). Membrane coating granules within these cells were often seen fusing with the lower layer of the stratum corneum to release their lipid contents (Figure 23). The stratum granulosum cells contained tonofilaments, damaged mitochondria, and vacuoles.

The cytoplasm of cells within the stratum spinosum was more compact than that of cells within the stratum granulosum and contained numerous tonofilament bundles, glycogen, and mitochondria. Occasional electron dense compact masses seen within the cells were probably the cross-section of tonofilament bundles.

The stratum basale cells were cuboidal to slightly elliptical. Although the basal cells appeared fairly normal, a few cells did contain small vacuoles, nuclear envelope separation, random rather than subbasal mitochondria which also had blown out cristae, large cytoplasmic vacuoles, and thickened tonofilaments (Figures 24 and 25). Moderate intercellular epidermal

edema between basal cells was common and desmosomes between adjacent basal cells appeared to have large dense plaques but no tonofilaments.

Hemidesmosomes were very numerous along the plasma membrane of these basal cells (Figures 24 and 25). Although a broken, slightly amorphous zone similar to the lamina lucida and lamina densa was discerned beneath the hemidesomosomes, no "true" basement membrane was actually present. This "pseudo basement membrane" was relatively flat, not highly convoluted like the basement membrane of normal human skin. Focal areas of the basement membrane beneath the hemidesmosomes did appear to have fine anchoring filaments. However, anchoring fibrils and microfibril bundles were not identified between the basement membrane and the dermis (Figure 26). The underlying collagen-rich dermis was acellular (Figure 26). In one section, the upper 20% of the dermis appeared devoid of any collagen matrix. A layer of an amorphous ground substance containing vacuoles very similar to that normally present within basal intercellular spaces was situated between the pseudo basement membrane area and the dermal collagen matrix (Lot# 312C; Figure 27).

The Day 1 samples were similar to the Day 0 samples. The bottom layer of the stratum corneum had more extensive stratum corneum projections down into the stratum granulosum. The prominent stratum granulosum contained rounded keratohyalin granules and thick tonofilament bundles. Within the basal cells, chromatin clumping and differentiation were seen. Fine fibrils appeared to originate and align parallel to the "pseudo basement membrane" area.

The stratum corneum of Day 2 samples remained compact and consisted of up to 19 cell layers. Within the viable epidermis, tonofilaments were thicker and desmosome connections between adjacent cells had thicker and denser attachment plaques. In some areas of the basement membrane microvesicles were seen. There was an increase in collagen fibrils (Figure 28) along the basement membrane.

Reduced cellular integrity in the Day 3 samples indicated an overall cellular degeneration of the culture. The stratum corneum appeared less organized due to fewer

desmosomes and possessed fewer compact cellular layers. The stratum granulosum contained remnants of mitochondria and fewer membrane coating granules. Also, nuclear margination and slight nuclear envelope separation were more common. Occasional stratum basale cells were vacuolated. Some of the large vacuoles may represent ruptured mitochondria that have coalesced to form these paranuclear vacuoles. Tonofilaments were not as thick or dense and fewer hemidesmosomes were located along the basal cell membrane. In several areas, there was an increase in the fine fibrils that were oriented parallel to the basement membrane area. As noted in a single Day 0 sample (Lot# 312C), a thick layer of an amorphous ground substance was present between the basement membrane area and dermal collagen (Lot# 311B; Figure 29).

ADVANCED TISSUE SCIENCES

Light Microscopy: The HSE from Advanced Tissue Sciences is a three dimensional culture in which fibroblasts are grown on a nylon mesh combined with human keratinocytes to form a stratified epidermis. This model consisted of a compact stratum corneum, a stratum granulosum layer, a stratum spinosum layer, a stratum basale layer, with a thick extracellular matrix (dermis) containing numerous fibroblasts and cross-sections of nylon fibers (Figure 30). The fibroblasts immediately surrounding the fibers of nylon mesh were slightly distorted, particularly the cells of the epidermis which were usually compressed immediately above the fiber. Therefore, only areas in between the nylon fibers were evaluated. The stratum corneum thickness measured 3-12μm in the Day 0 and Day 1 samples, 3-16μm in the Day 2 samples, and 3-22μm in the Day 3 samples. The majority of the stratum corneum was normal in all samples, with the exception of one Day 3 (EM133) where the cell layers were very thick, undifferentiated, and therefore unrecognizable. While cells within the stratum granulosum and stratum spinosum layers were flat, the cells of the stratum basale were cuboidal to elliptical. Focal separation at the basement membrane area were found in all samples from Day 0 (Figure 31) through Day 3.

Epidermal thickness was fairly consistent within the Day 0 and Day 1 samples, ranging between 14-45μm. The overall epidermal thickness and variability within samples increased in Day 2 (20-50μm) and Day 3 (39-58μm) cultures except for one Day 3 sample (EM133) were it measured only 14μm thick and contained areas of general necrosis. No rete pegs were present in any of the Day 0 through Day 3 samples. Morphological integrity was highly variable within the epidermis, with areas of focal (Lot#s 1472-091493-138K-20E and 1481-111693-138K-7D) and widespread (Lot#s 1478-102693-138K-13E and 1480-110993-104K-15B) necrosis. All Day 0, Day 1, Day 2 (Figure 32), and Day 3 (Figure 33) samples exhibited an increase in dark basal cells, vacuoles, cellular necrosis, and epidermal-dermal separation. Intercellular edema within the stratum spinosum and stratum basale layers of Day 0 lots was highly variable, ranging from moderate (Lot#s 1472-091493-138K-20E and 1481-111693-

138K-7D) to severe (Lot#s 1478-102693-138K-13E and 1480-110993-104K-15B). The dermis of Day 0 through Day 3 samples was averaged 63μm thick and encompassed each nylon fiber. Fibroblasts were more numerous in the dermis of Day 3 samples.

Electron microscopy: The stratum corneum of the Day 0 samples were well organized into compact parallel layers (up to 12 layers thick) and connected by desmosomes. Membrane coating granules, usually retained within the lower layers of the stratum corneum, resembled vacuoles. An occasional epidermal cell was retained within the stratum corneum. The upper most layers of the stratum corneum (stratum disjunctum) were less compact and often contained microvillus-like processes primarily on the outermost plasmalemma of the stratum corneum layer (Figure 34). In addition, some stratum corneum cells were not completely keratinized and had retained their tonofilaments.

The flattened cells of the stratum granulosum were connected by intermittent desmosomes, fine and sparse tonofilaments oriented parallel to the cells and few keratohyalin granules. Membrane coating granules were present in the upper stratum granulosum and often seen fused to the lower stratum corneum layer. The vacuoles noted in some cells of the upper stratum granulosum and between the stratum corneum and stratum granulosum layers probably represents degenerative organelles or membrane coating granules devoid of lipid (Figure 35). Periodically, upper stratum granulosum and lower stratum corneum cells were not properly differentiated and possessed fine granular material, possibly cross-sections of tonofilaments (Figure 36).

The stratum spinosum cell layers were flattened rather than polyhedral and connected to adjacent cells via numerous desmosomes. The cells of the stratum spinosum contained Golgi, mitochondria, and an increase in tonofilaments in addition to focal areas completely devoid of cytoplasm (Figure 37). Areas of epidermal necrosis were focal (Lot#s 1472-091493-138K-20E and 1481-111693-138K-7D) to widespread, with a reduction or complete loss of the stratum spinosum layer (Lot#s 1478-102693-138K-13E and 1480-110993-104K-15B; Figure

38). Intercellular epidermal edema, highly variable within lots (intralot), was moderate to severe as noted by light microscopy.

The cuboidal to elliptical cells of the stratum basale contained adequate mitochondria, rough endoplasmic reticulum, well-developed Golgi, sparse tonofilaments, a few lipid droplets, and sparse desmosome attachments. Also, the stratum basale cells contained a few small vacuoles and autophagic vacuoles (Figure 39). An occasional necrotic cell or dyskeratotic basal cell consisting of thick tonofilaments, vacuoles, and microvesicles was noted (Figure 40). Intercellular basal edema was prominent in most Day 0 samples. Endocytotic invaginations were present along the periphery of the basal cell, with coated and uncoated pinocytotic vesicles within the cell cytoplasm (Figure 41).

The basement membrane was absent along much of the basal cell plasma membrane, including areas where processes of the basal cells extended into the dermis (Figures 42). In areas lacking a basement membrane, empty spaces were often found in the dermis immediately below the basal cell plasma membrane (Figures 42 and 43). Hemidesmosomes were present but not numerous along the basal plasma membrane. Anchoring filaments were seen in the lamina lucida beneath the hemidesosomes. Anchoring fibrils (Figure 43) and microfibrils were present along the fragmented lamina densa and extended into the superficial dermis.

The dermis of Day 0 samples contained necrotic (Figure 44) and vacuolated fibroblasts as well as fibroblasts in various stages of differentiation. These cells were found intermittently within the collagen-rich dermal matrix and along its lower seed layer. The cells within this lower seed layer contained numerous organelles, including dense whorls, vacuoles, Golgi, pinocytotic vesicles, and rough endoplasmic reticulum (Figure 45).

The Day 1 samples were similar to Day 0, with microvillus processes arising from the stratum disjunctum and vacuoles within the stratum corneum layers. The stratum granulosum and stratum spinosum layers contained an increased number of vacuoles with focal areas of necrosis. In addition, the stratum spinosum and stratum basale cells contained some necrotic cells that consisted of tonofilament whorls, blown out mitochondria, and chromatin clumping

(Figure 46). Small inclusions with an electron dense border and an amorphous center were found within the stratum spinosum and stratum basale intercellular spaces resembling cross sections of an extended cellular process that contacted an adjacent cell. The basement membrane zone at Day 1 was identical to that of Day 0, including the empty spaces within the dermis immediately below the basal plasma membrane.

The Day 2 samples consisted of epidermal cells in different stages of cellular degeneration. This degeneration within the epidermis consisted of necrotic cells containing blown out mitochondria and lysosomes, as well as cells with an electron lucent cytoplasm and dark basal cells (Figure 47). In areas lacking a basement membrane, empty spaces in the dermis were present like the Day 0 and Day 1 samples (Figure 48). Necrotic fibroblasts were noted in the upper dermis, with cellular debris below the loosely packed seed layer.

The Day 3 cultures exhibited a dramatic increase in cellular necrosis. While the stratum spinosum and stratum basale layers within one lot were completely necrotic (EM132), another lot had samples with focal areas of necrosis and a non-differentiated stratum corneum (EM133; Figure 49). In addition, this sample exhibited little cellular organization within the viable epidermis and vacuoles were numerous within all cell layers. The remaining Day 3 samples (EM131) had areas of electron lucent cytoplasm in the stratum granulosum as well as blown out mitochondria and several autophagic vacuoles within the stratum spinosum. The stratum basale cells contained swollen and blown out mitochondria, lysosomes, and membranous whorls. Basal cell vacuoles were present in the area of the lamina lucida and the lamina densa. (Figure 50). Otherwise, the discontinuous basement membrane zone was identical to the Day 0, Day 1, and Day 2 samples.

DISCUSSION

The objective of this study was to evaluate the morphology between two commercially available *in vitro* human skin equivalents (HSEs) and natural human skin (NHS) as models for future evaluations with bis-2-chloroethyl sulfide (HD). This study ultrastructurally assessed natural human skin (NHS) and HSEs manufactured by Mat Tek (MT) and Advanced Tissue Sciences (ATS) to determine interlot and intralot variability.

Although the general morphology of the NHS and the HSEs at the light microscopic level appeared similar, specific anatomical differences were present (Table 3). In general, all three consisted of a stratified squamous epithelium made up of a typical stratum corneum, stratum granulosum, stratum spinosum, and stratum basale cell layers supported by a dermal matrix. The compact stratum corneum was smooth in the HSEs and convoluted in the NHS. The stratum corneum thickness of all NHS samples was fairly consistent. The stratum corneum of the HSEs was thicker and more variable, which increased with culture storage time. The stratum corneum layers of the HSEs retained numerous cellular remnants and were not always completely keratinized (MT and ATS) or differentiated (ATS). The stratum corneum of all MT cultures sent numerous fingerlike projections between the cells of the stratum granulosum. The outermost plasma membrane of the upper stratum corneum (stratum disjunctum) of some ATS samples retained microvillus-like processes which probably played a role in nutrient absorption.

The cells within the viable epidermis of the HSEs differed somewhat in shape from those of the NHS. The HSEs possessed cuboidal to elliptical shaped cells in the stratum basale layer rather than the typical cuboidal to columnar. The epidermis of all NHS and MT samples during all culture storage times did fall within specific thickness ranges, but the overall epidermal thickness and variability of the ATS samples increased with storage time. The epidermal cells of both *in vitro* cultures contained typical organelles such as membrane coating granules (stratum granulosum), nuclei, nucleoli, mitochondria, endoplasmic reticulum, Golgi apparatus, lipid droplets, and tonofilaments. Arrangement of mitochondria in the stratum

basale of MT samples appeared random rather than concentrated beneath the nuclei. Desmosomes in the MT and ATS samples were sparse compared to NHS. The numerous keratohyalin granules within the stratum granulosum of NHS were stellate, more rounded in the MT samples and practically absent in the ATS samples. Melanocytes (Lot#s 9310C253 and 9310C277) and Langerhans cells were present in NHS and but absent within the HSEs. Therefore, the shape, organization, differentiation, distribution and frequency of these organelles did vary between the NHS and HSE cultures.

The degree of intercellular edema in the stratum spinosum and stratum basale of NHS was consistent within (intralot) but not between (interlot) lots. The Mat Tek HSE had occasional intracellular edema within the stratum granulosum (Lot# 312B) and moderate intercellular edema in the stratum basale (Lot# 312C). Intralot variablilty was high within three ATS lots: Lot# 1478-102693-138K-13E, slight to severe; Lot# 1480-110993-104K-15B, slight to severe; Lot# 1481-111693-138K-7D, none to moderate. Areas of focal (Lot#s 1472-091493-138K-20E and 1481-111693-138K-7D) and widespread (Lot#s 1478-102693-138K-13E) necrosis were present. Based on the severity of epidermal necrosis and intercellular edema, the quality of Lot# 1478-102693-138K-13E and Lot# 1480-110993-104K-15B was inferior.

The basement membrane of both HSEs, unlike that of the NHS, was smooth due to the physical culture constraints and did not possess the epidermal rete pegs present in NHS. An underdeveloped basement membrane zone was present in both HSEs. Although the ATS culture did produce a basement membrane typical of NHS, it was present in less than 50% of the available area. In areas lacking the basement membrane, focal spaces devoid of dermal elements were found. Unlike the microvesicles formed in NHS in response to chemical insult, these spaces did not involve the basement membrane. The MT HSE had a poorly developed "pseudo basement membrane" that lacked a true lamina lucida and lamina densa as well as the accompanying anchoring filaments, anchoring fibrils and microfibril bundles. Occasional microvesicles were present along this "pseudo basement membrane" in the Day 2 samples.

The dermis of all cultures consisted of a collagen-rich matrix, denoted by numerous collagen fibers and/or fibrils. The dermis of NHS typically contained numerous cell types (fibroblasts, macrophages, mast cells, etc.) and site specific adnexial structures (sebaceous glands, sweat glands, hair follicles, etc.). The dermis of ATS samples contained fibroblasts that were often vacuolated or necrotic, while that of MT was completely acellular. When the seed layer appeared healthy, the epidermal cells were not; when the epidermis was healthy, the seed cell layer was inferior.

Cellular degeneration increased in the NHS from Day 3 to Day 7 and in the HSEs from Day 1 to Day 3. This was generally characterized by an increase in vacuoles, nuclear envelope separation, swollen and blown out mitochondria, and dark basal cells, and later the appearance of autophagic vacuoles, dyskeratotic cells, and necrotic cells. As with the Day 0 samples, the cellular degeneration was much greater in the ATS HSE.

In conclusion, the overall epidermal integrity of the Day 0 NHS and MT cultures was good. That was not true, however, for the Day 0 ATS cultures. Widespread necrosis and severe intercellular epidermal edema were present in two of the four Day 0 lots assessed. The remaining lots contained focal necrosis and moderate epidermal edema. This, in addition to the vacuolization within the stratum corneum and epidermis, the presence of spaces within the dermal matrix, and the discontinuous basement membrane compromised the integrity of this HSE. Utilizing the ATS HSE to study HD dermatotoxicity, which causes these types of effects within normal skin models, would be very difficult. Within the MT HSE, the "pseudo basement membrane" may not respond to HD toxicity with the formation of classical microvesicles since the lamina lucida and lamina densa were poorly developed. The toxic response of the MT and ATS HSEs to HD must be determined in the Phase II Dose Response Study. Normal human skin, MT, and ATS HSEs stored beyond Day 0 exhibited an increase in epidermal necrosis, vacuolization, blown out mitochondria, and dark basal cells and should not be used to assess HD dermatotoxicity. To minimize any possible interlot and/or intralot variability found within the NHS and the HSEs, it is essential that all samples exposed to HD

be accompanied by a corresponding control. As this study illustrates, the MT and ATS *in vitro* models do mimick NHS but have specific limitations, especially in regards to the basement membrane zone.

FUTURE RESEARCH DIRECTIONS:

One of the greatest issues of concern between NHS and the HSEs is the morphological and antigenic characterization of the basement membrane. The basement membrane is extremely important because it is susceptible to injury by chemical vesicants such as bis-2 (chloroethyl sulfide) (sulfur mustard) and dichloro(2-chlorovinyl)arsine (lewisite). Therefore, further characterization of the basement membrane should be performed. The basement membrane epitopes of these HSEs should be mapped to determine if they are antigenically similar to that of NHS. Our laboratory routinely performs indirect immunofluorescence and indirect immunoelectron microscopy on basement membrane epitopes such as laminin, type IV collagen, bullous pemphigoid antibody, epidermolysis bullosa acquisita antibody, fibronectin, and GB_3 monoclonal antibody. These future studies would determine important similarities or differences between these HSEs and NHS.

Table 1: Summary of all tissue samples processed for transmission electron microscopy.

DATE RECEIVED	BATTELLE SAMPLE NO.	NCSU TEM SAMPLE NO.	SAMPLE DESCRIPTION
7/10/93	EM 1	112-93	Mat Tek Lot 300A Day 0 Sample 1
	EM 2	113-93	Sample 2
	EM 3	114-93	Sample 3
	EM 4	115-93	Lot 300B Day 0 Sample 1
	EM 5	116-93	Sample 2
	EM 6	117-93	Sample 3
	EM 7	118-93	Lot 300D Day 0 Sample 1
	EM 8	119-93	Sample 2
	EM 9	120-93	Sample 3
	EM 10	121-93	Lot 300E Day 0 Sample 1
	EM 11	122-93	Sample 2
	EM 12	123-93	Sample 3
	EM 13	124-93	Mat Tek Lot 300A Day 1 Sample 4
	EM 14	125-93	Sample 5
	EM 15	126-93	Sample 6
	EM 16	127-93	Day 2 Sample 7
	EM 17	128-93	Sample 8
	EM 18	129-93	Sample 9
	EM 19	130-93	Day 3 Sample 10
	EM 20	131-93	Sample 11
	EM 21	no tissue	Sample 12
	EM 22	132-93	ATS Lot 1461A-062293-104K Day 0 Sample 1
	EM 23	133-93	Sample 2
	EM 24	134-93	Sample 3
8/5/93	EM 25	135-93	Lot 104K-1462-070693 Day 0 Sample 1
	EM 26	136-93	Sample 2
	EM 27	137-93	Sample 3
8/18/93	EM 28	146-93	Lot 104K-1463-071393 Day 0 Sample 1
	EM 29	147-93	Sample 2
	EM 30	148-93	Sample 3
	EM 31	149-93	Day 1 Sample 1
	EM 32	150-93	Sample 2
	EM 33	151-93	Sample 3
	EM 34	152-93	Day 2 Sample 1
	EM 35	153-93	Sample 2
	EM 36	154-93	Sample 3

DATE RECEIVED	BATTELLE SAMPLE NO.	NCSU TEM SAMPLE NO.	SAMPLE DESCRIPTION
8/18/93	EM 37	155-93	ATS Lot 104K-1463-071393 Day 3 Sample 1
	EM 38	156-93	Sample 2
	EM 39	157-93	Sample 3
8/19/93	EM 40	158-93	Lot 104K-1465-072793 Day 0 Sample 1
	EM 41	159-93	Sample 2
	EM 42	160-93	Sample 3
	EM 43	161-93	Lot 104K-1464-072093 Day 2 Sample 1
9/21/93	EM 44	168-93	Mat Tek Lot 311B Day 0 Sample 1
	EM 45	169-93	Sample 2
	EM 46	170-93	Sample 3
	EM 47	171-93	Lot 271 Day 0 Sample 1
	EM 48	172-93	Sample 2
	EM 49	173-93	Sample 3
	EM 50	174-93	Lot 311B Day 1 Sample 1
	EM 51	175-93	Sample 2
	EM 52	176-93	Sample 3
	EM 53	177-93	Day 2 Sample 1
	EM 54	178-93	Sample 2
	EM 55	179-93	Sample 3
	EM 56	180-93	Day 3 Sample 1
	EM 57	181-93	Sample 2
	EM 58	182-93	Sample 3
9/29/93	EM 59	183-93	Lot 312B Day 0 Sample 1
	EM 60	184-93	Sample 2
	EM 61	185-93	Sample 3
	EM 62	186-93	Lot 312C Day 0 Sample 1
	EM 63	187-93	Sample 2
	EM 64	188-93	Sample 3
10/15/93	EM 65	197-93	ATS Lot 1472-091493-138K-20E Day 0 Sample 1
	EM 66	198-93	Sample 2
	EM 67	199-93	Sample 3
11/2/93	EM 68	200-93	NHS ID# 9310C253 Day 0 Sample 1
	EM 69	201-93	Sample 2
	EM 70	202-93	Sample 3
	EM 71	203-93	ID# 9310C277 Day 0 Sample 1
	EM 72	204-93	Sample 2
	EM 73	205-93	Sample 3
	EM 74	206-93	Day 3 Sample 1
	EM 75	207-93	Sample 2
	EM 76	208-93	Sample 3

DATE RECEIVED	BATTELLE SAMPLE NO.	NCSU TEM SAMPLE NO.	SAMPLE DESCRIPTION
11/2/93	EM 77	209-93	NHS ID# 9310C277 Day 5 Sample 1
	EM 78	210-93	Sample 2
	EM 79	211-93	Sample 3
	EM 80	212-93	Day 7 Sample 1
	EM 81	213-93	Sample 2
	EM 82	214-93	Sample 3
11/17/93	EM 83	215-93	ID# 9311C053 Day 0 Sample 1
	EM 84	216-93	Sample 2
	EM 85	217-93	Sample 3
11/30/93	EM107	226-93	ID# 9311C104R Day 0 Sample 1
	EM108	227-93	Sample 2
	EM109	228-93	Sample 3
11/30/93	EM113	229-93	ATS Lot 1478-102693-138K-13E Day 0 Sample 1
	EM114	230-93	Sample 2
	EM115	231-93	Sample 3
12/21/93	EM116	232-93	NHS ID# 9312C031R Day 0 Sample 1
	EM117	233-93	Sample 2
	EM118	234-93	Sample 3
	EM119	235-93	ATS Lot 1480-110993-104K-15B Day 0 Sample 1
	EM120	236-93	Sample 2
	EM121	237-93	Sample 3
	EM122	238-93	Lot 1481-111693-138K-7D Day 0 Sample 1
	EM123	239-93	Sample 2
	EM124	240-93	Sample 3
	EM125	241-93	Day 1 Sample 1
	EM126	242-93	Sample 2
	EM127	243-93	Sample 3
	EM128	244-93	Day 2 Sample 1
	EM129	245-93	Sample 2
	EM130	246-93	Sample 3
	EM131	247-93	Day 3 Sample 1
	EM132	248-93	Sample 2
	EM133	249-93	Sample 3

Table 2: Specific tissue samples assessed by transmission electron microscopy.

Natural Human Skin - 21 samples

ID# 9310C277			
Day 0 EM68	Day 3 EM74	Day 5 EM77	Day 7 EM80
EM69	EM75	EM78	EM81
EM70	EM76	EM79	EM82

ID # 9310C253	ID#9311C053	ID#9311C104R
Day 0 EM71	Day 0 EM83	Day 0 EM107
EM72	EM84	EM108
EM73	EM85	EM109

Mat Tek - 21 samples

Lot 311B			
Day 0 EM44	Day 1 EM50	Day 2 EM53	Day 3 EM56
EM45	EM51	EM54	EM57
EM46	EM52	EM55	EM58

Lot 271	Lot 312B	Lot 312C
Day 0 EM47	Day 0 EM59	Day 0 EM62
EM48	EM60	EM63
EM49	EM61	EM64

Advanced Tissue Science - 21 samples

1481-111693-138K-7D			
Day 0 EM122	Day 1 EM125	Day 2 EM128	Day 3 EM131
EM123	EM126	EM129	EM132
EM124	EM127	EM130	EM133

1472-091493-138K-20E	1478-102693-138K-13E	1480-110993-104K-15B
Day 0 EM65	Day 0 EM113	Day 0 EM119
EM66	EM114	EM120
EM67	EM115	EM121

Table 3: Comparison of Natural Human Skin, Mat Tek HSE, and Advanced Tissue Sciences HSE.

Morphology		NHS	MT	ATS
Stratum corneum		convoluted	smooth	smooth
thickness	D-0	6-11μm	19μm	3-12μm
	D-1	N/A	25μm	3-12μm
	D-2	N/A	27-56μm	3-16μm
	D-3	6-11μm [1]	47-64μm	3-22μm
Epidermis				
rete pegs[1]		present	absent	absent
thickness	D-0	45-65μm [2]	80-130μm	14-45μm
	D-1	N/A	80-130μm	14-45μm
	D-2	N/A	80-130μm	20-50μm
	D-3	45-65μm [1,2]	80-130μm	39-58μm
cell shape	SG[1]	flat	circular-flat	flat
	SS[1]	polyhedral	polyhedral	flat
	SB[1]	cuboidal-columnar	cuboidal-elliptical	cuboidal-elliptical
melanocytes[1]		present	absent	absent
Langerhans cells[1]		present	absent	absent
intercellular				
edema	SS[1]	none-severe	none-moderate	none-severe
	SB[1]	none-severe	none-moderate	none-severe
Basement membrane[1]		convoluted	smooth	smooth
hemidesmosomes		numerous	more numerous	less numerous
lamina lucida		continuous	"pseudo"	discontinuous
lamina densa		continuous	"pseudo"	discontinuous
anchoring filaments		present	present	present
anchoring fibrils		present	absent	present
microfibrils		present	absent	present
Dermis[1]				
thickness		>2000μm [3]	3-8μm	63μm
fibroblasts		numerous[4]	absent	numerous
collagen		numerous fibers	numerous fibrils	numerous fibers
adnexial structures		present	absent	absent

D-0 (Day 0), D-1 (Day 1), D-2 (Day 2), D-3 (Day 3); SG (stratum granulosum), SS (stratum spinosum), SB (stratum basale).
[1]Identical with increased culture storage times.
[2]Measurements excluded rete peg areas.
[3]Biopsies included only partial dermis.
[4]Additional cell types were observed.

FIGURES

Figure 1. Light micrograph showing melanocytes (arrows) in stratum basale layer. Note epidermis (E), dermis (D), and residual culture medium (CM). NHS, Day 0, 380X

Figure 2. Light micrograph depicting severe intercellular epidermal edema (arrows). Note stratum corneum (SC), vacuoles (V) in basal cells, and residual culture medium (CM). NHS, Day 0, 480X

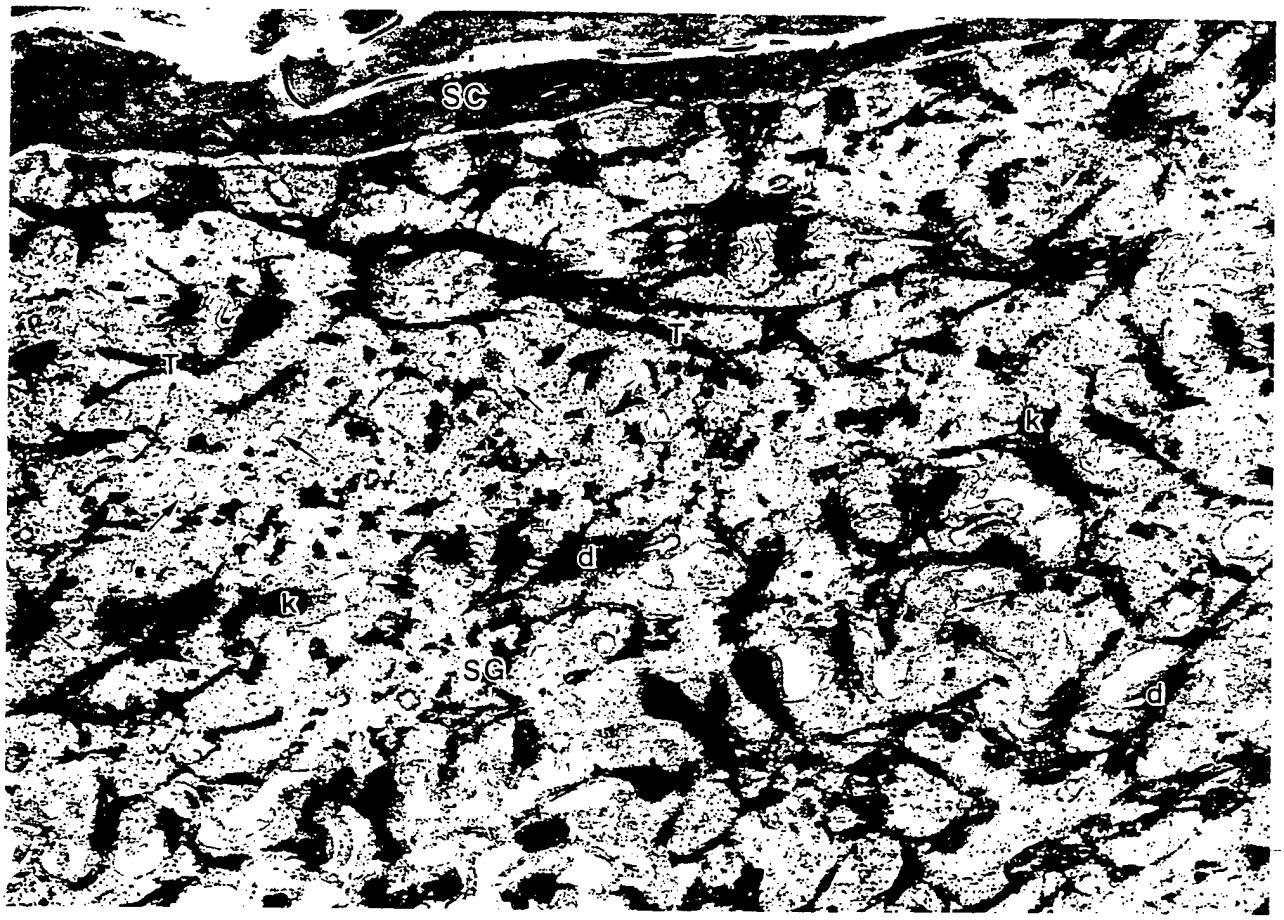

Figure 3. Transmission electron micrograph showing membrane coating granules fusing (large arrows) to the lower stratum corneum (SC) layer. Note normal stratum granulosum (SG), membrane coating granules (small arrows), desmosomes (d), tonofilaments (T), and keratohyalin granules (k). NHS, Day 0, 19,070X

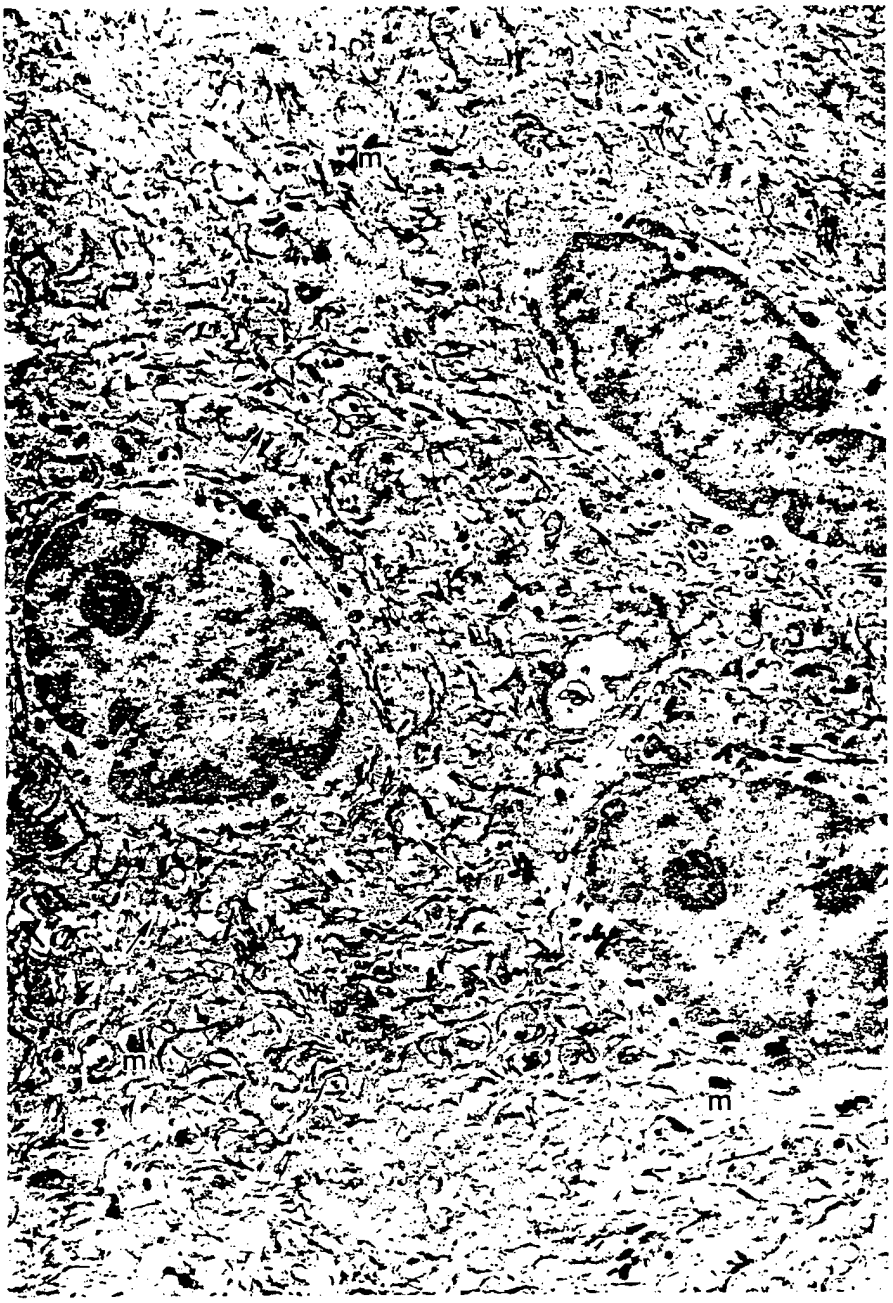

Figure 4. Transmission electron micrograph of stratum spinosum and stratum basale layers with slight intercellular edema (arrows). Note melanosomes (m). NHS, Day 0, 7,500X

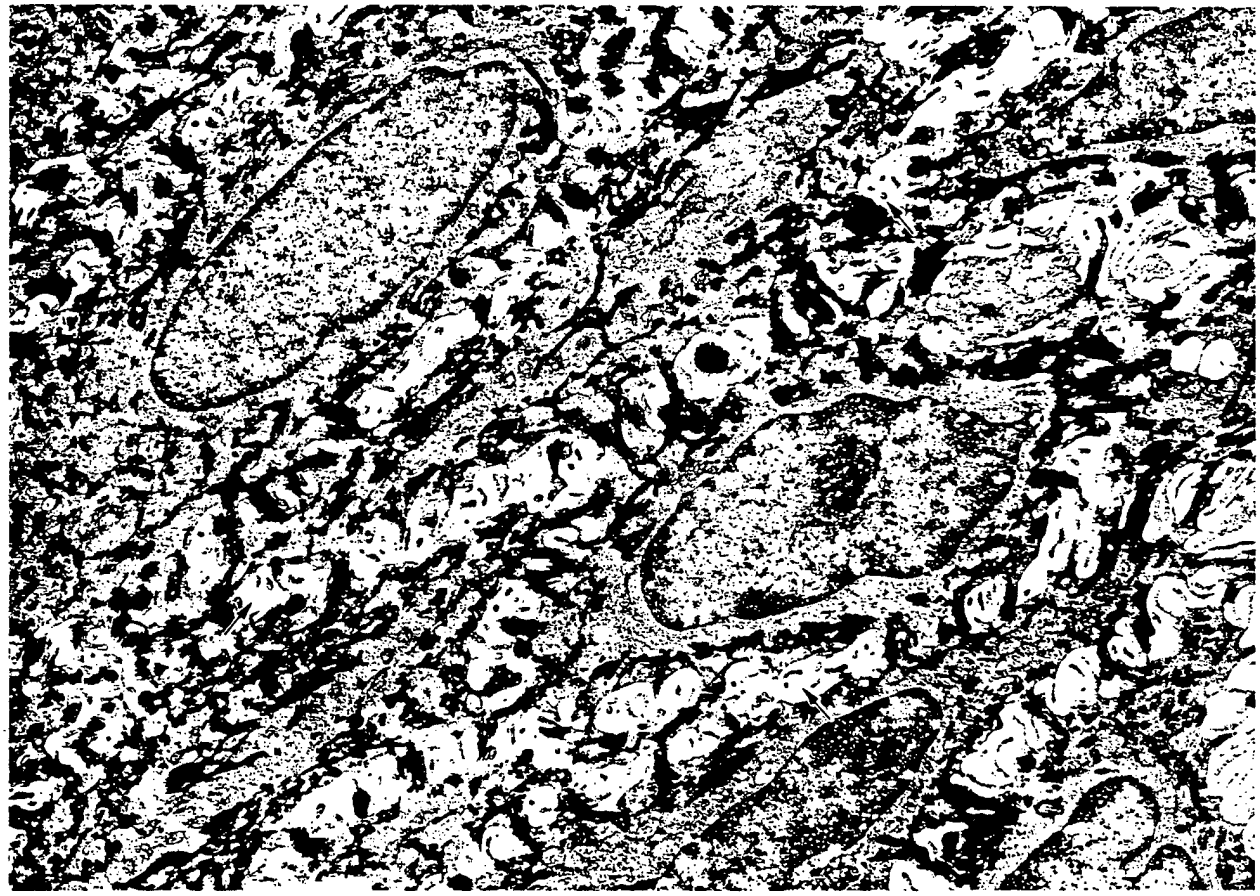

Figure 5. Transmission electron micrograph of the stratum spinosum layer with moderate intercellular edema (arrows). NHS, Day 0, 8,950X

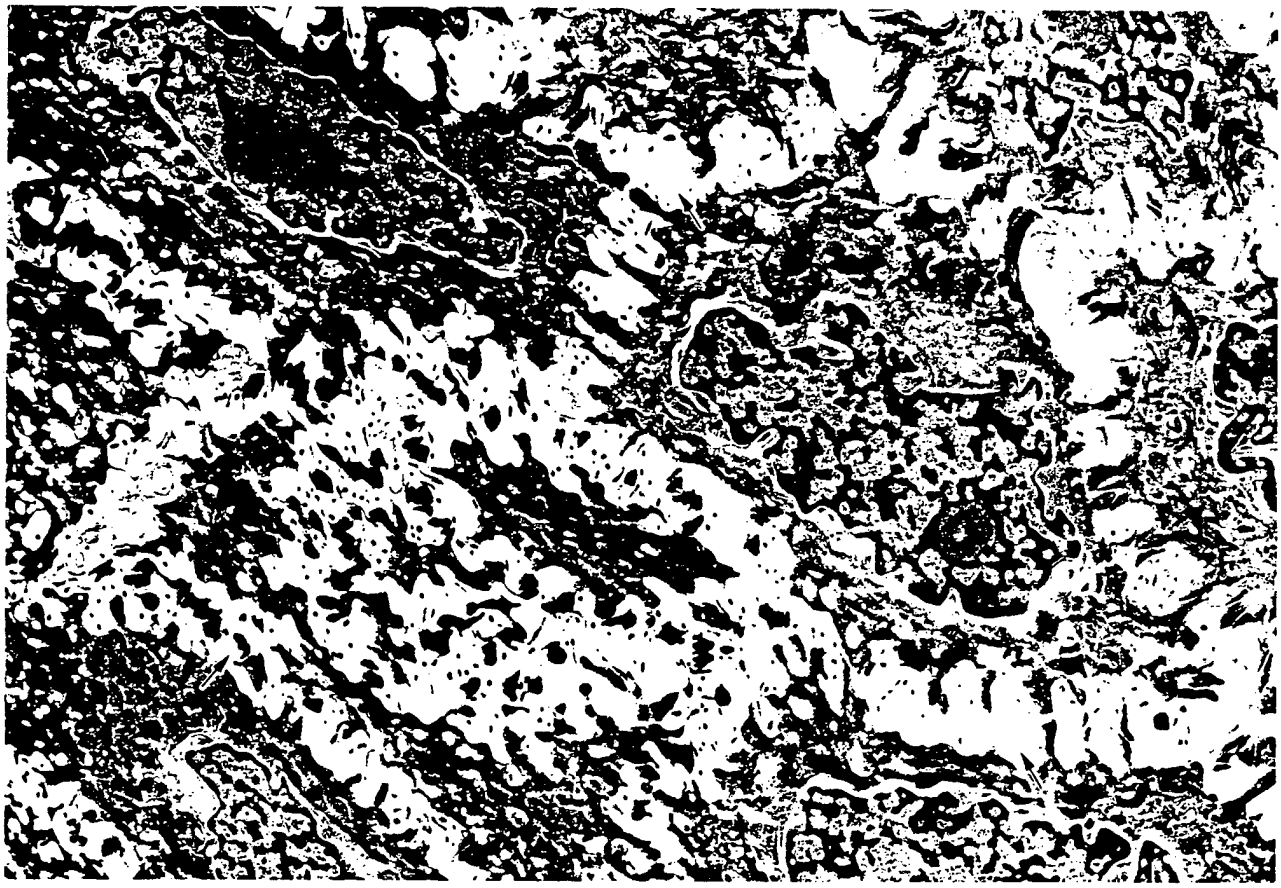

Figure 6. Transmission electron micrograph of the stratum spinosum layer with severe intercellular edema (arrows). NHS, Day 0, 8,950X

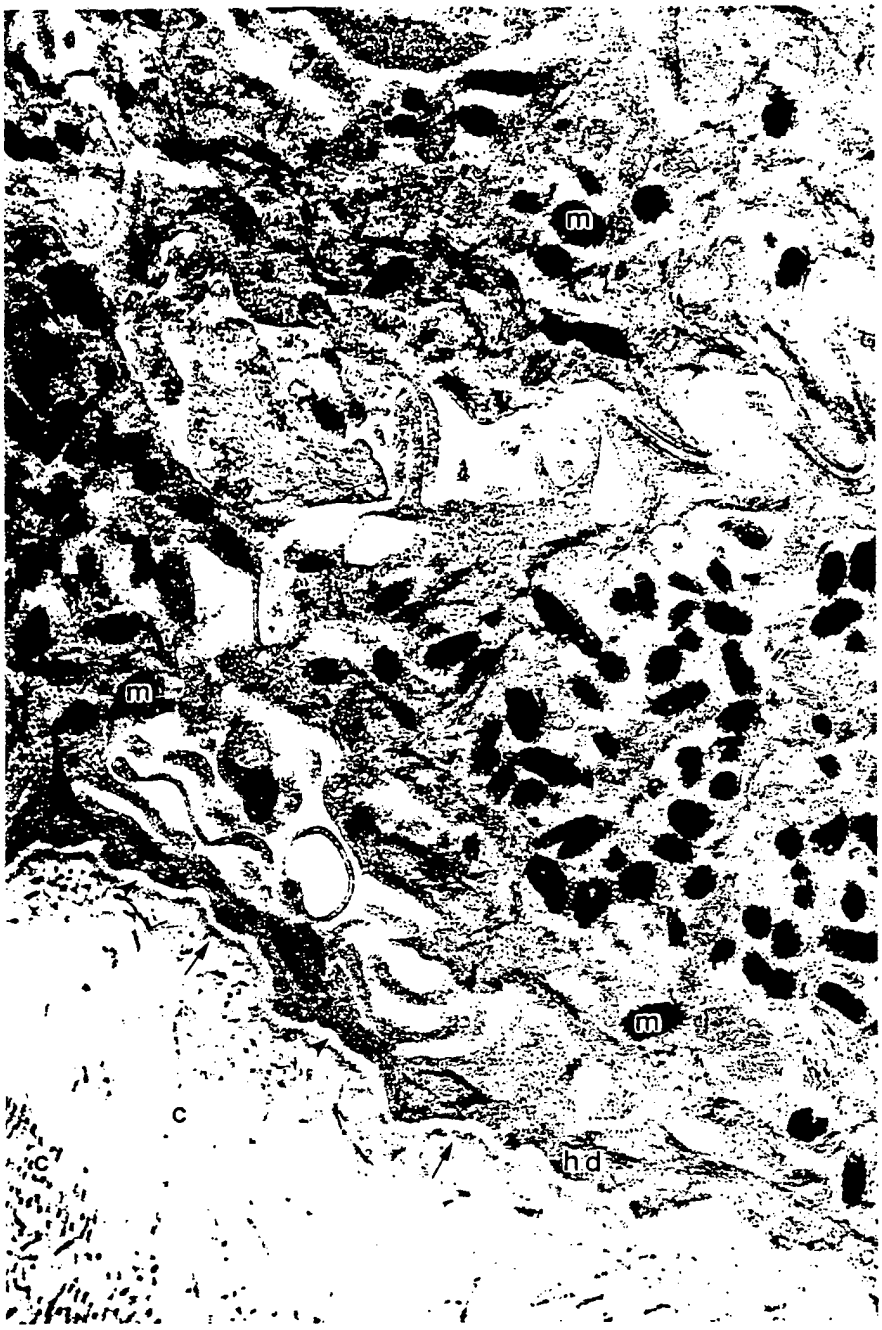

Figure 7. Transmission electron micrograph of stratum basale cells containing stage IV melanosomes (m). Note hemidesmosomes (hd), lamina lucida (arrowheads), lamina densa (arrows), and dermal collagen (c). NHS, Day 0, 26,000X

Figure 8. Transmission electron micrograph showing a normal basement membrane. Note hemidesmosomes (hd), lamina lucida (LL), lamina densa (LD), anchoring filaments (arrowheads), anchoring fibrils (small arrows), microfibril bundles (large arrows), and collagen. NHS, Day 0, 51,000X

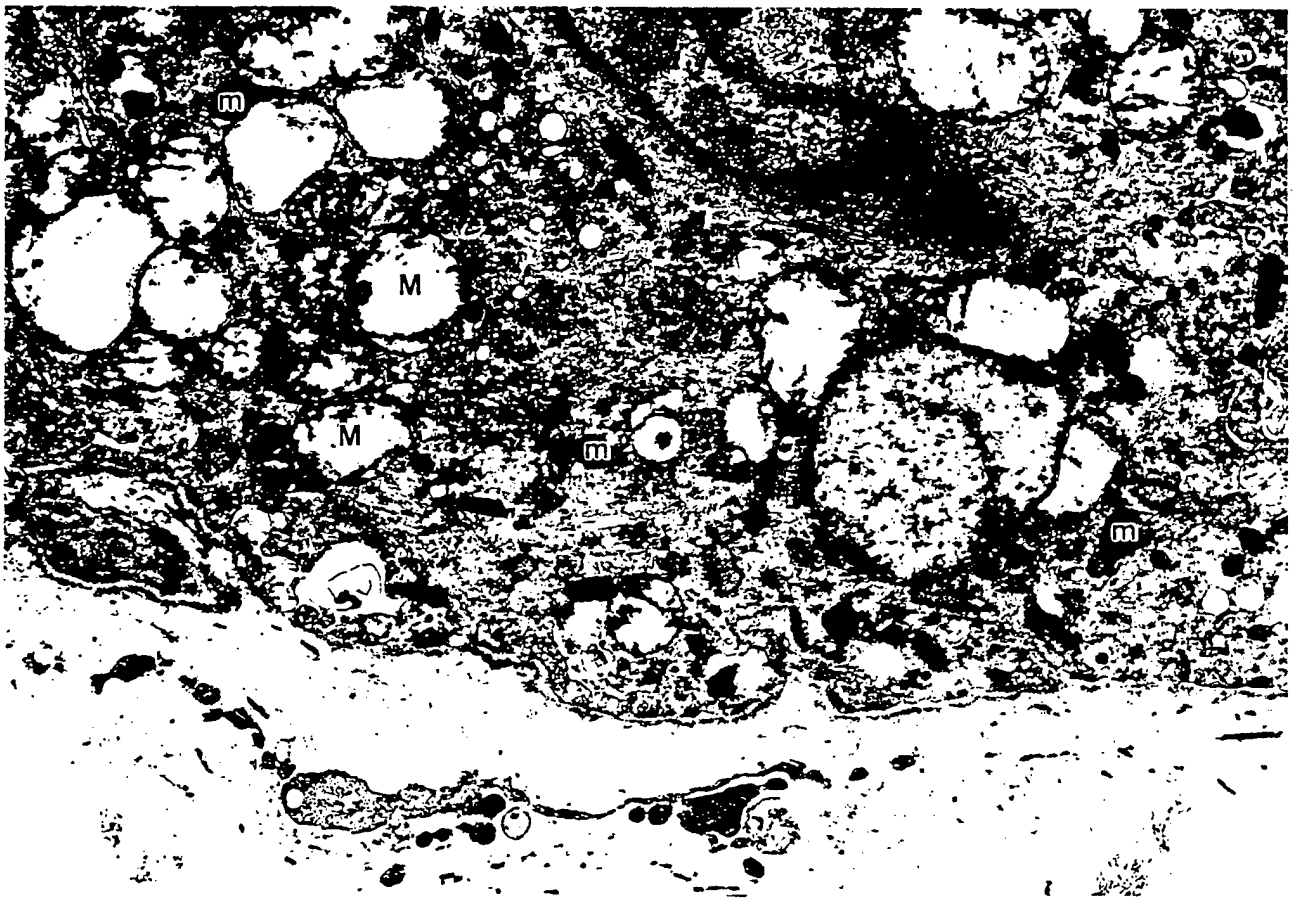

Figure 9. Transmission electron micrograph of a necrotic basal cell. Note ruptured mitochondria (M) and melanosomes (m). NHS, Day 3, 19,840X

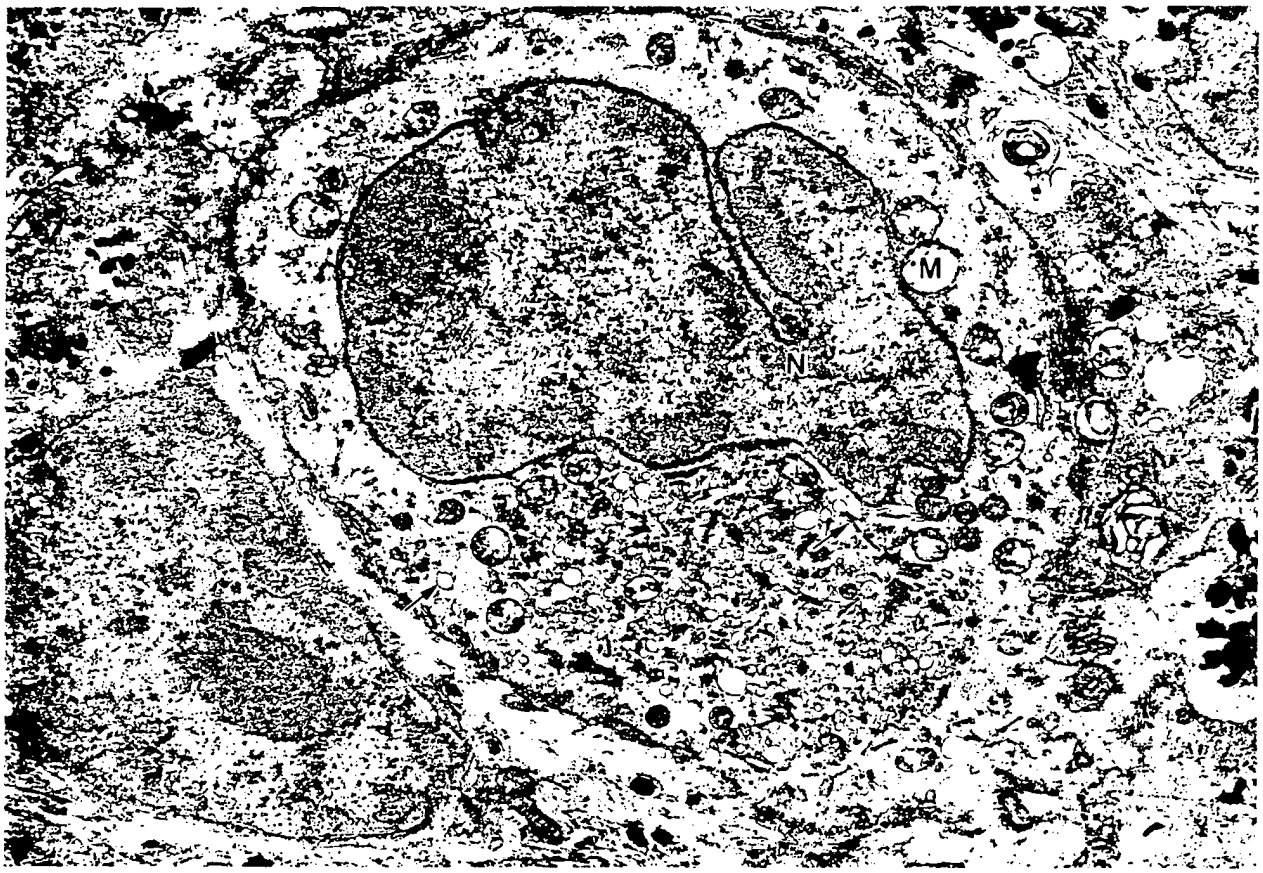

Figure 10. Transmission electron micrograph of a Langerhans cell within the stratum basale layer. Note the Langerhans cell granule (arrows), blown out mitochondria (M), indented nucleus (N), and the lack of tonofilaments and desmosomes. NHS, Day 3, 10,760X

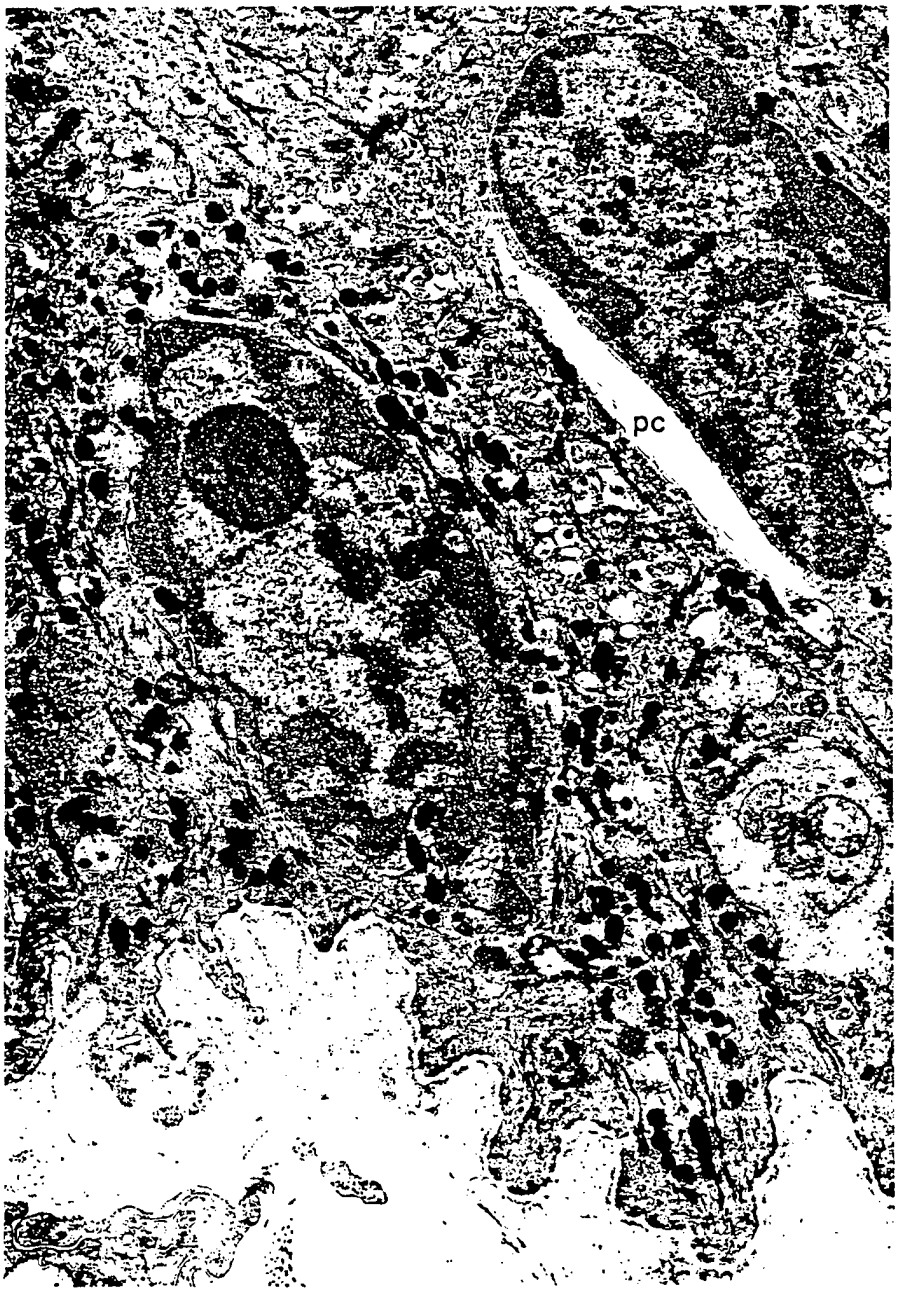

Figure 11. Transmission electron micrograph depicting a paranuclear cleft (pc). NHS, Day 3, 12,540X

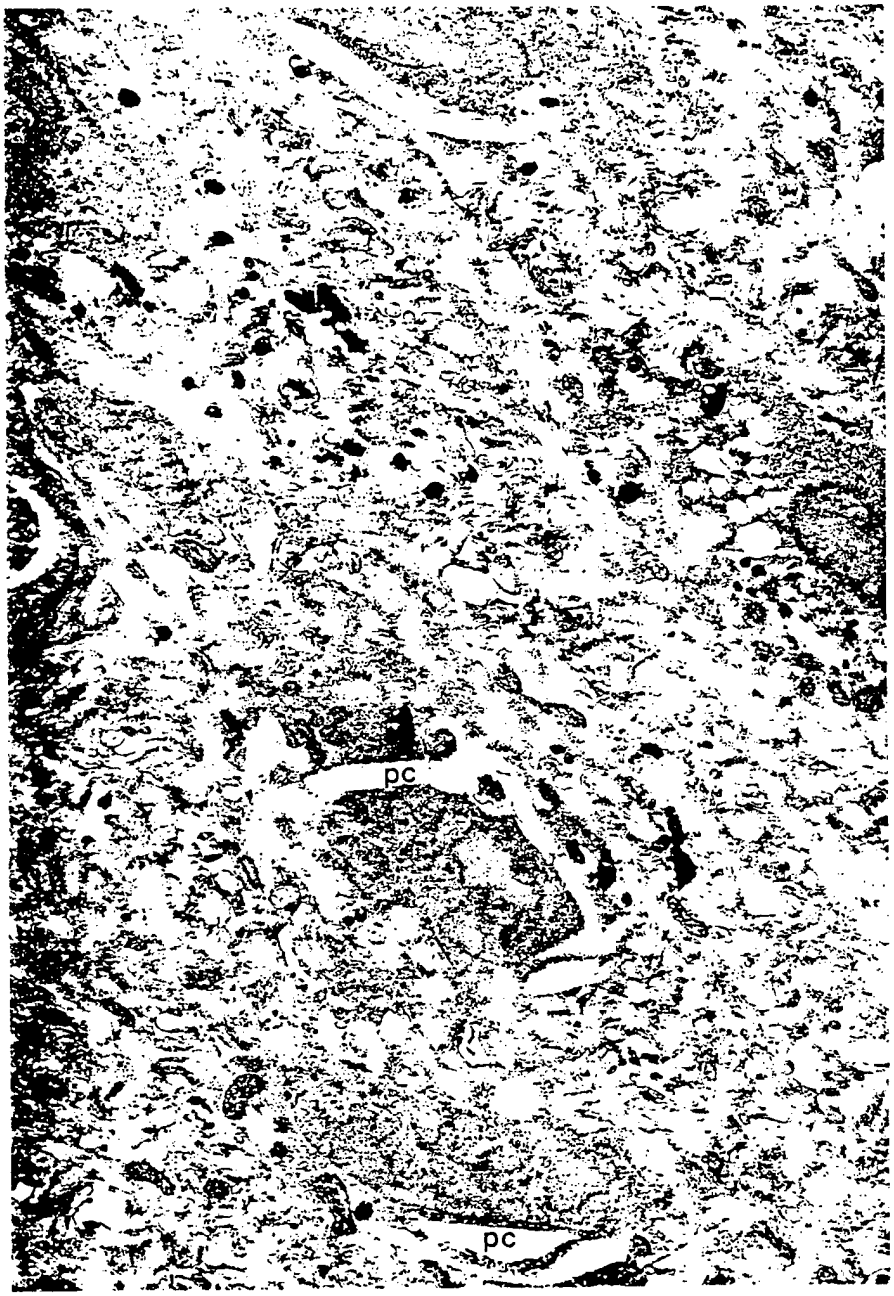

Figure 12. Transmission electron micrograph showing paranuclear clefts (pc) in the stratum basale and stratum spinosum cells. NHS, Day 5, 13,200X

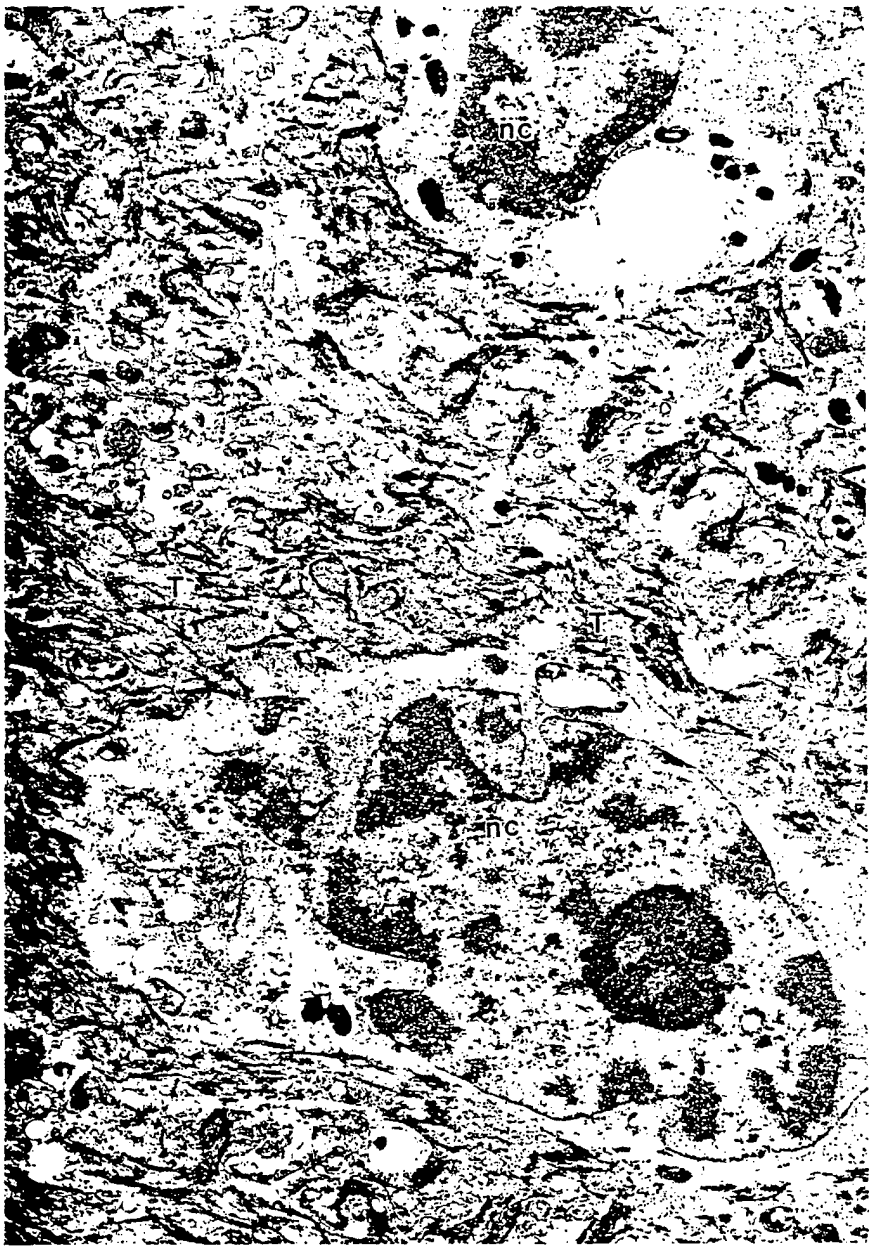

Figure 13. Transmission electron micrograph showing hyalinized tonofilaments (T) within the stratum spinosum layer. Note necrotic cells (nc). NHS, Day 7, 12,250X

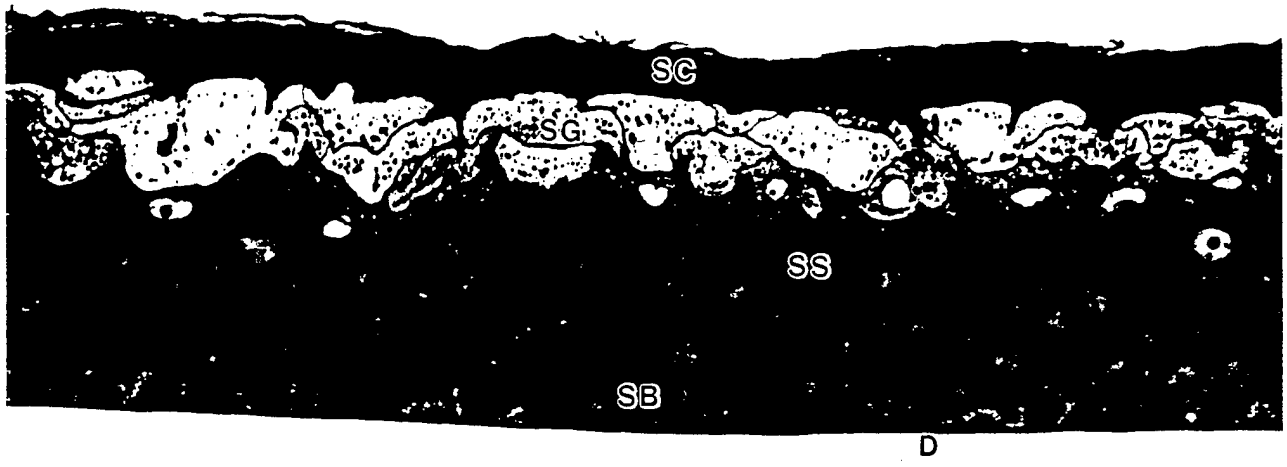

Figure 14. Light micrograph of an HSE showing a thick compact stratum corneum (SC), prominent stratum granulosum (SG), stratum spinosum (SS), stratum basale (SB), and acellular dermis (D). MT, Day 0, 580X

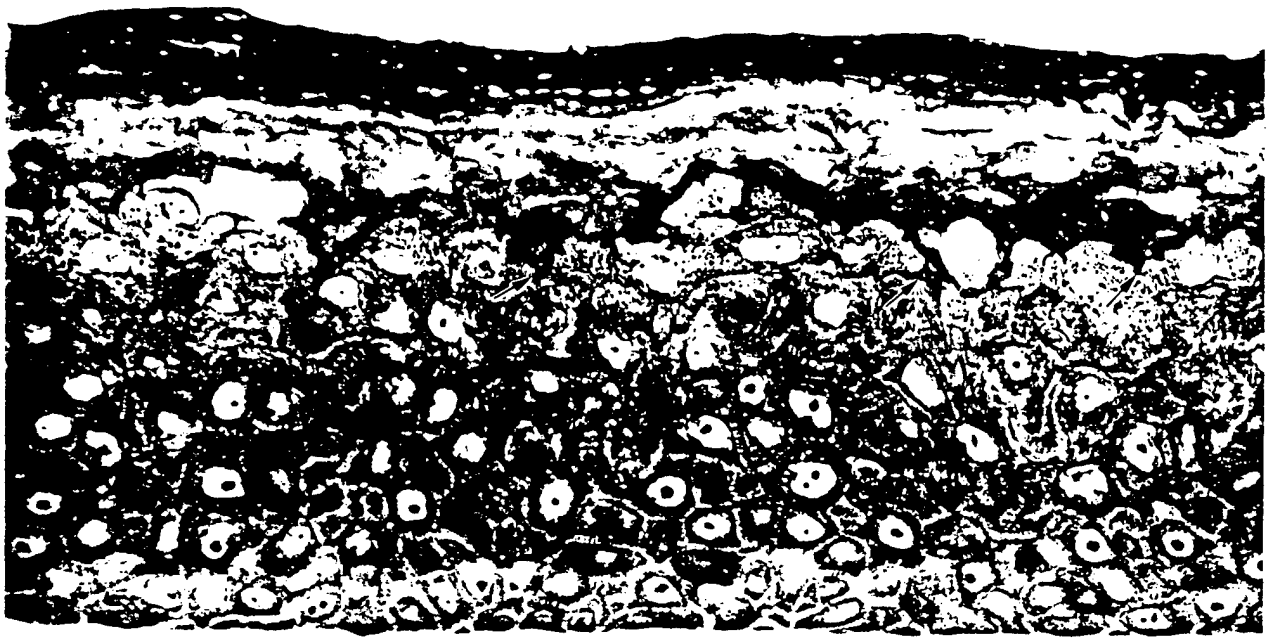

Figure 15. Light micrograph showing numerous fingerlike projections (arrows) of the stratum corneum extending between the cells of the stratum granulosum. MT, Day 0, 580X

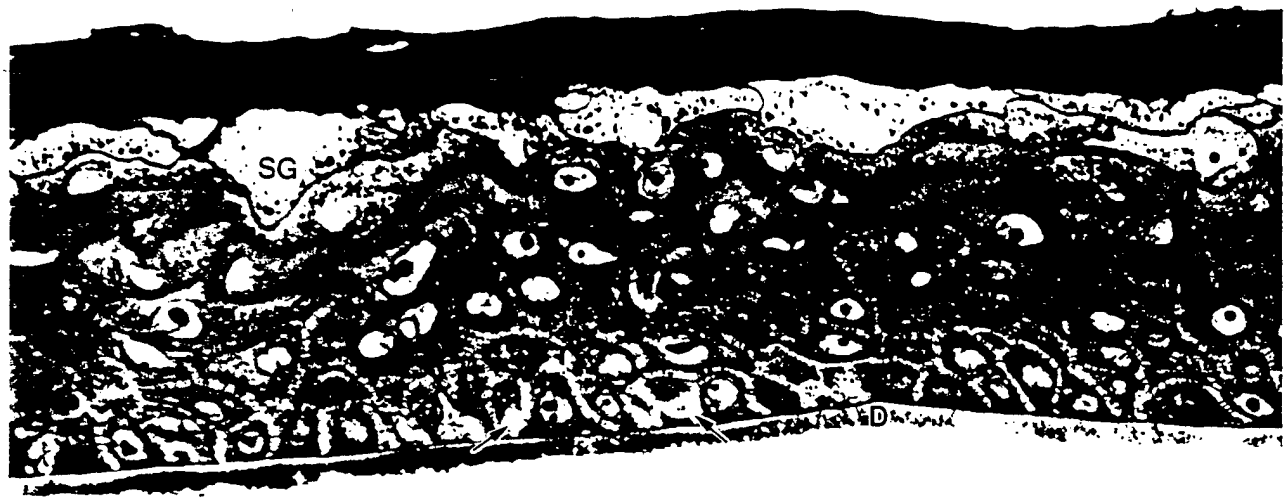

Figure 16. Light micrograph showing swollen stratum granulosum (SG) and basal intercellular edema (arrows). Note thin dermis (D) supporting the epidermis. MT, Day 0, 580X

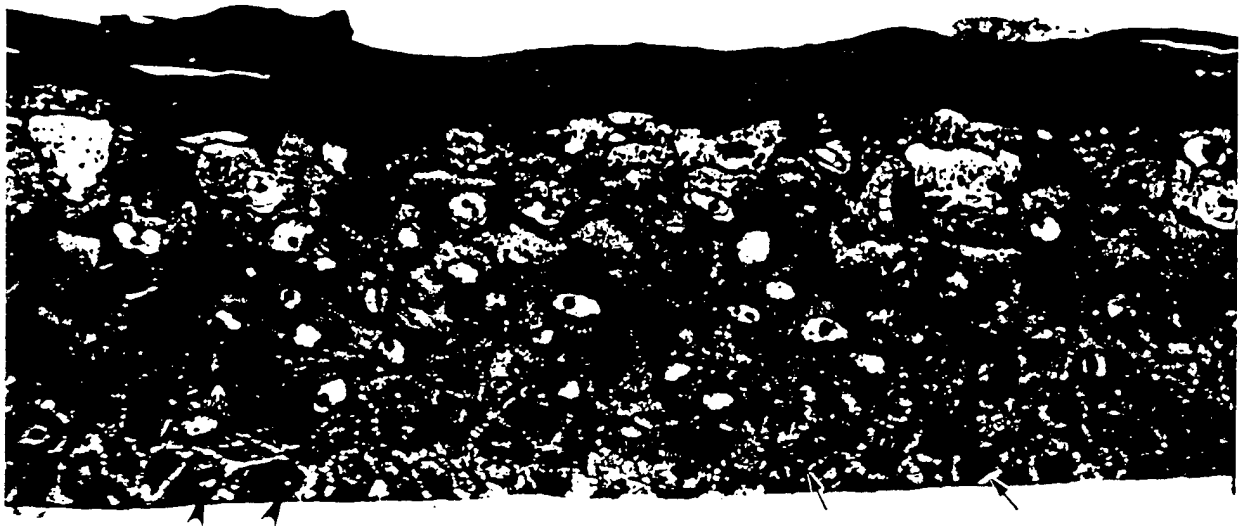

Figure 17. Light micrograph showing moderate intercelluar edema (arrows) within the stratum basale layer. Note vacuoles (arrowheads) in the stratum basale cells. MT, Day 0, 580X

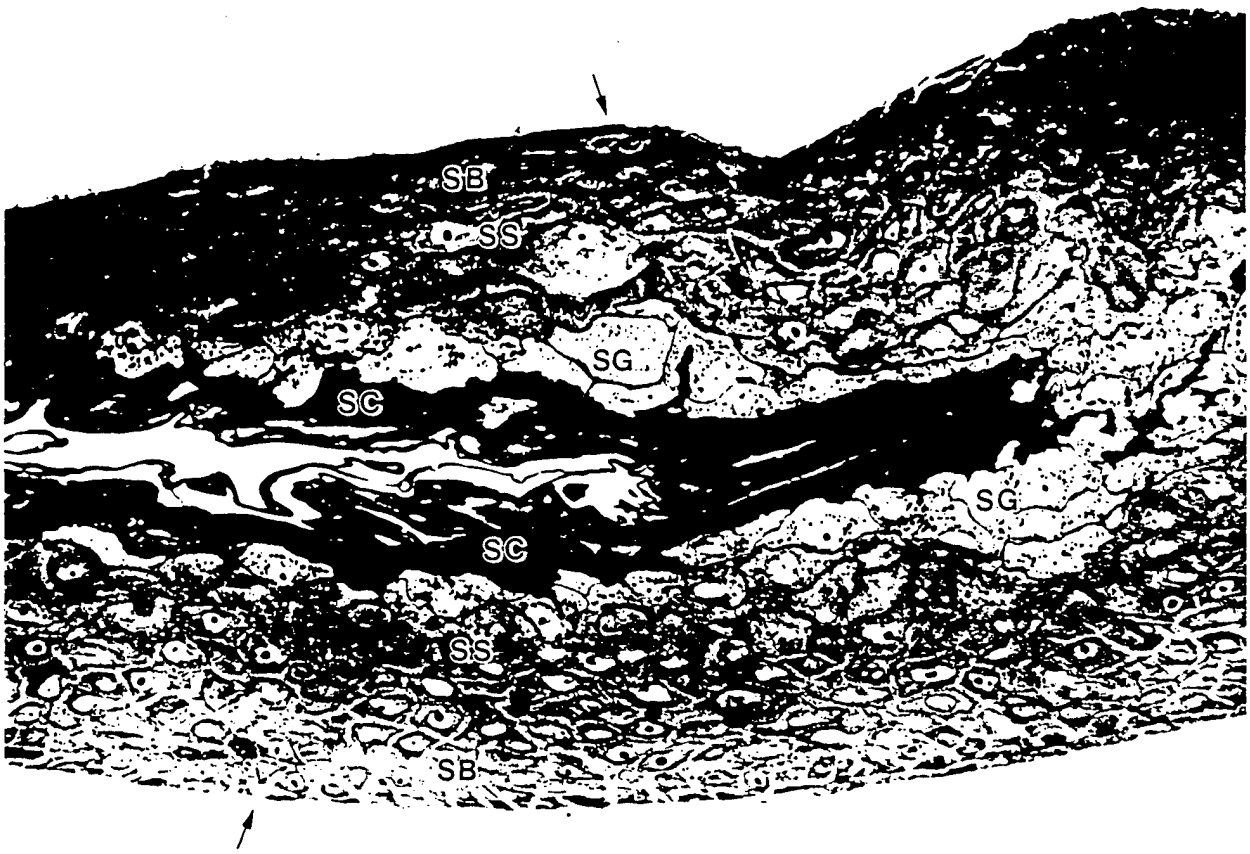

Figure 18. Light micrograph exhibiting abnormal stratification of the epidermis. Note stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), stratum basale (SB), and dermis (arrows). MT, Day 0, 400X

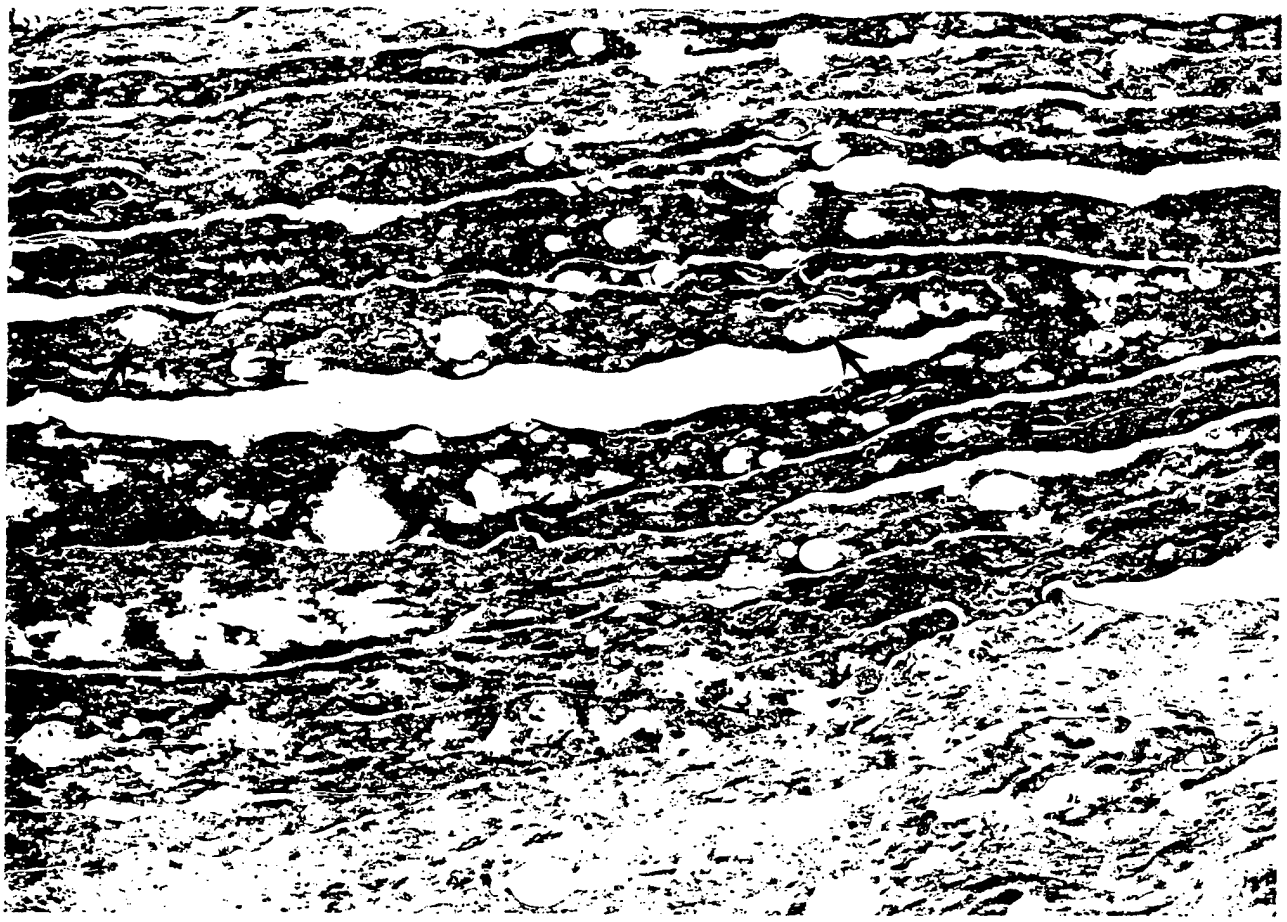

Figure 19. Transmission electron micrograph showing intracellular (large arrows) organelle remnants. MT, Day 0, 12,320X

Figure 20. Transmission electron micrograph showing intracellular (large arrows) and intercellular (small arrows) organelle remnants within the disorganized stratum corneum layers (SC). MT, Day 0, 4,840X

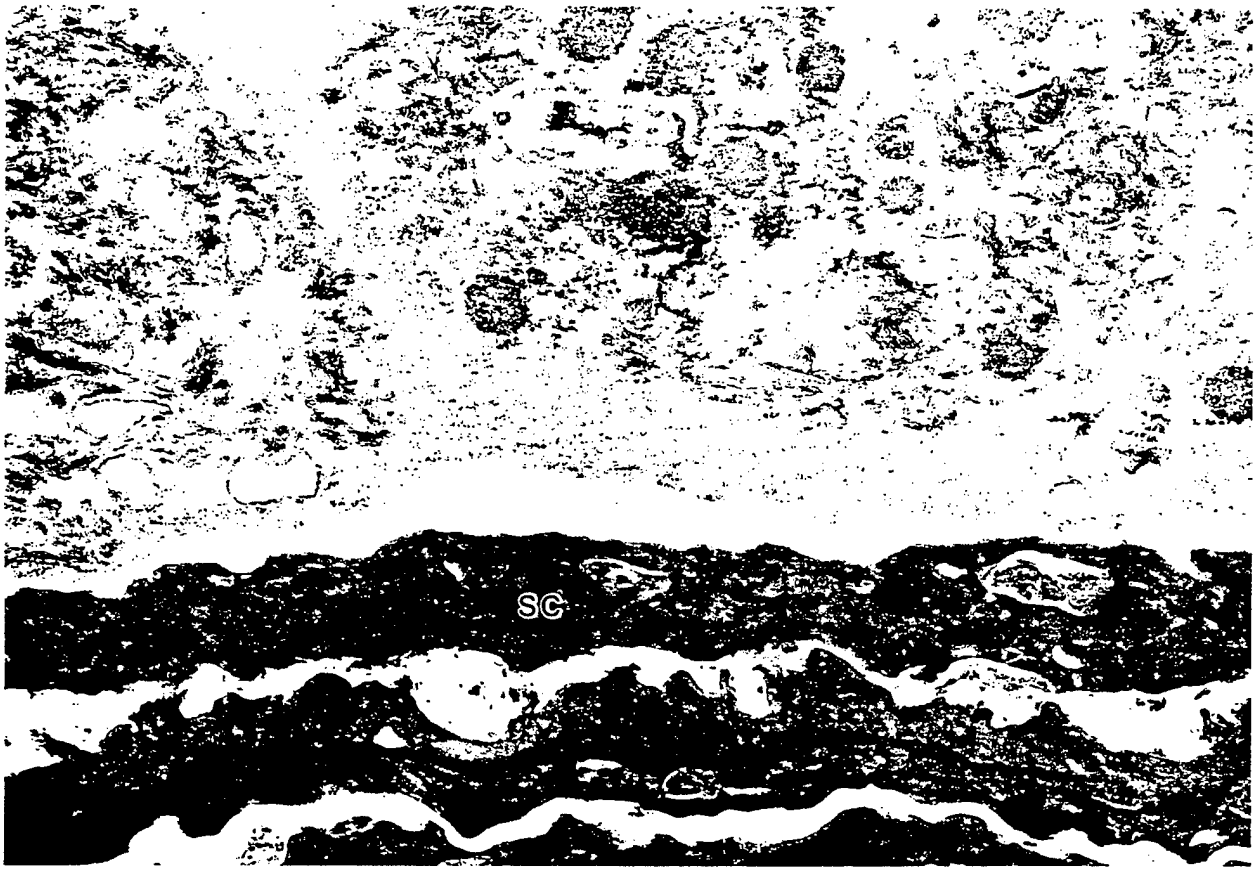

Figure 21. Transmission electron micrograph of an amorphous substance containing debris on the surface of the stratum corneum (SC). MT, Day 0, 21,950X

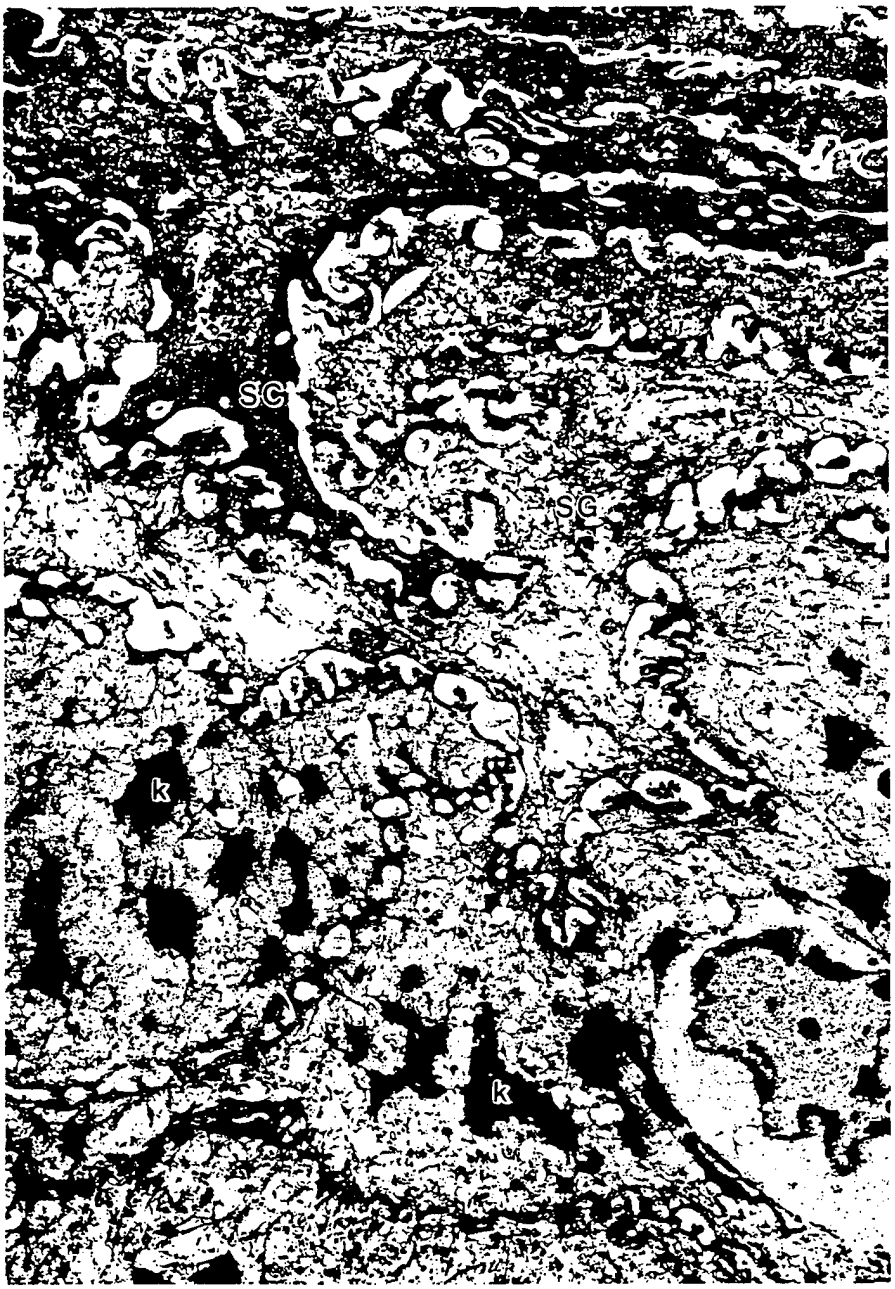

Figure 22. Transmission electron micrograph of the stratum corneum (SC) projecting into the stratum granulosum (SG) layer. Note keratohyalin granules (k). MT, Day 0, 5,950X

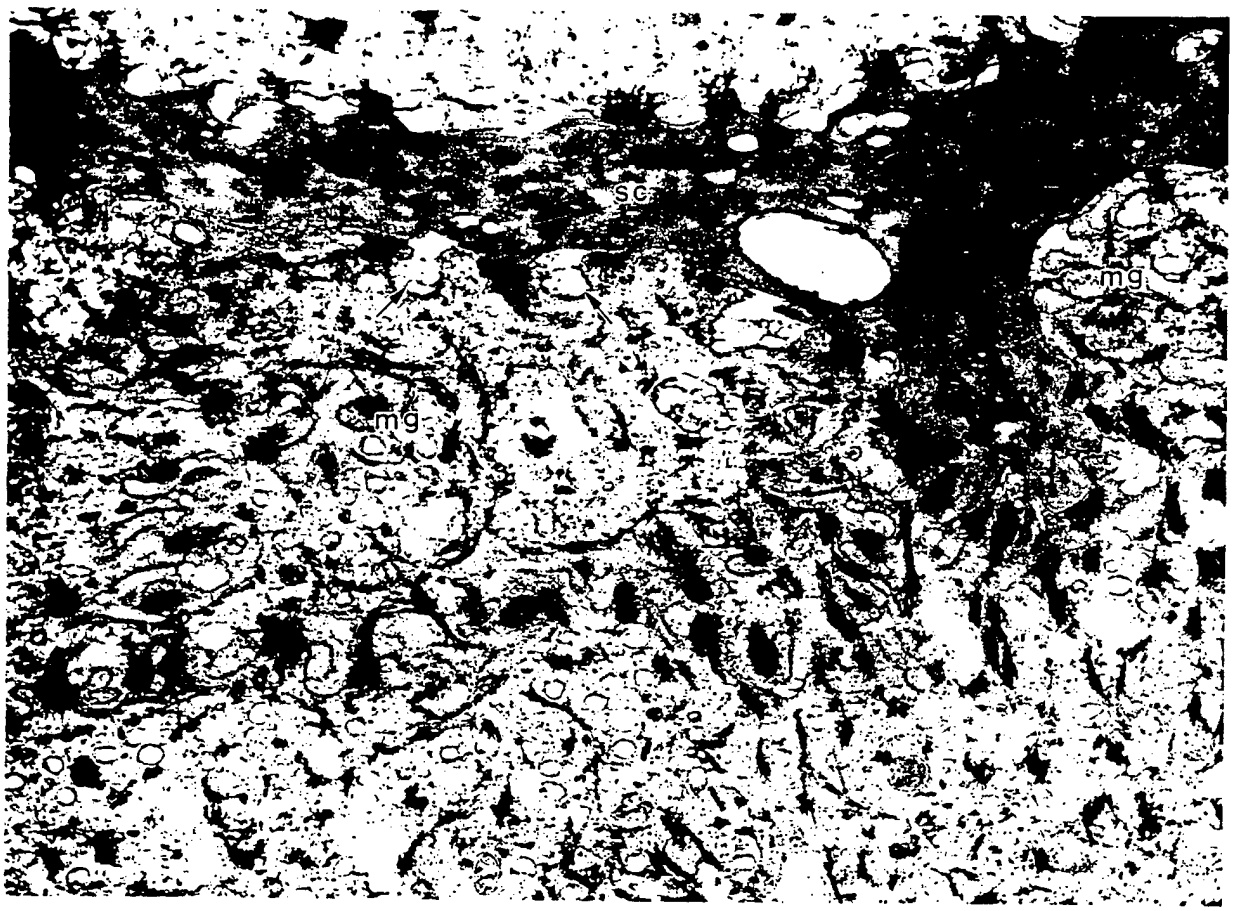

Figure 23. Transmission electron micrograph showing membrane coating granules (mg) fusing (arrows) to a stratum corneum projection (sc). MT, Day 0, 21,300X

Figure 24. Transmission electron micrograph showing normal stratum basale (SB) cells containing mitochondia (M), tonofilaments (T), desmosomes (d), vacuoles (V), and numerous hemidesmosomes (arrows) along the basement membrane. MT, Day 0, 11,880X

Figure 25. Transmission electron micrograph showing a damaged stratum basale (SB) cell. Note intercellular edema (arrows) and numerous hemidesmosomes (arrowheads). MT, Day 0, 13,200X

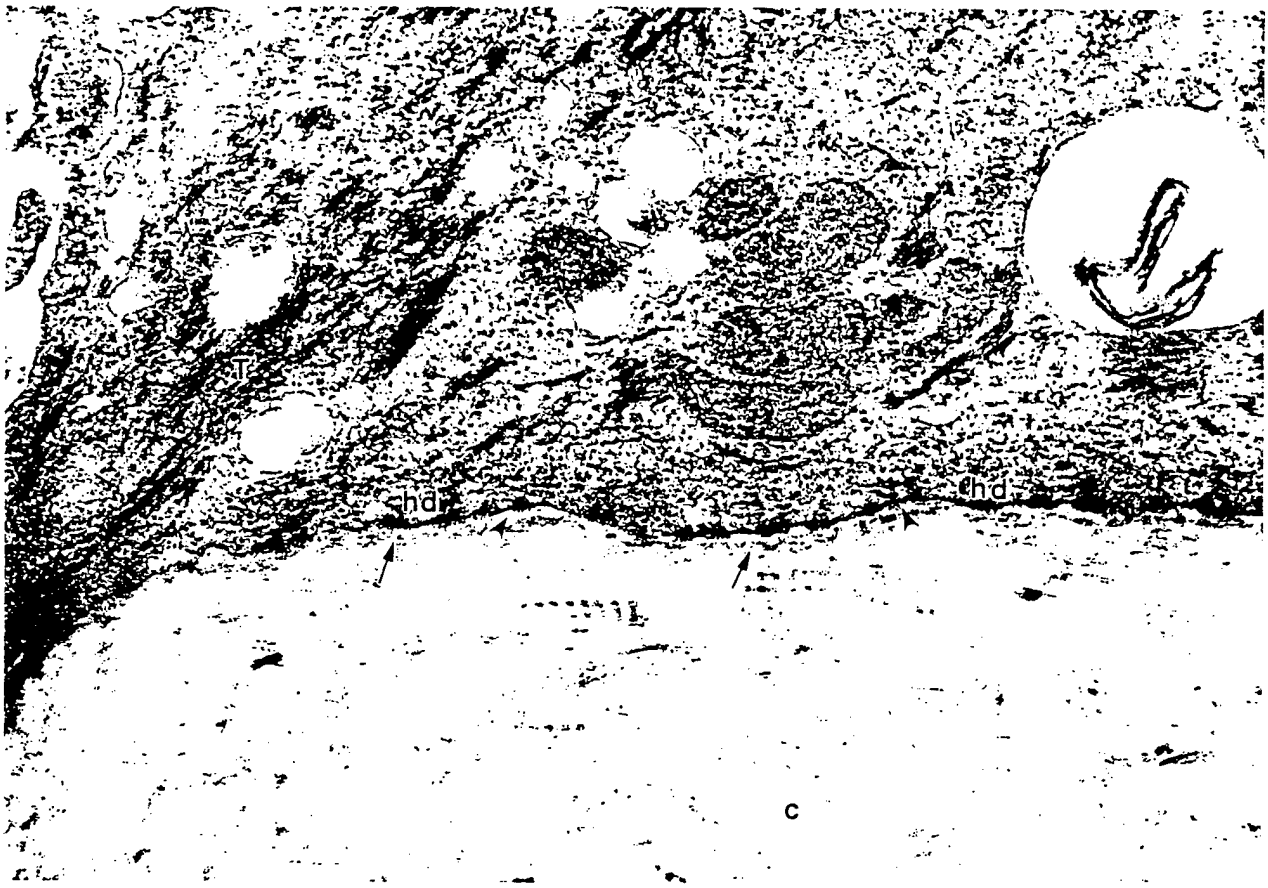

Figure 26. Transmission electron micrograph of the "pseudo basement membane". Note hemidesmosomes (hd), poorly developed lamina lucida (arrowheads) and lamina densa (arrows), tonofilaments (T), and dermal collagen (c). MT, Day 0, 41,600X

Figure 27. Transmission electron micrograph of an amorphous ground substance containing vacuoles (V) formed between the basement membrane (arrows) and dermal collagen (c). MT, Day 0, 26,000X

Figure 28. Transmission electron micrograph of the "pseudo basement membrane". Note hemidesmosomes (hd), poorly developed lamina lucida (arrowheads) and lamina densa (arrows), and dermal collagen (c). MT, Day 2, 114,000X

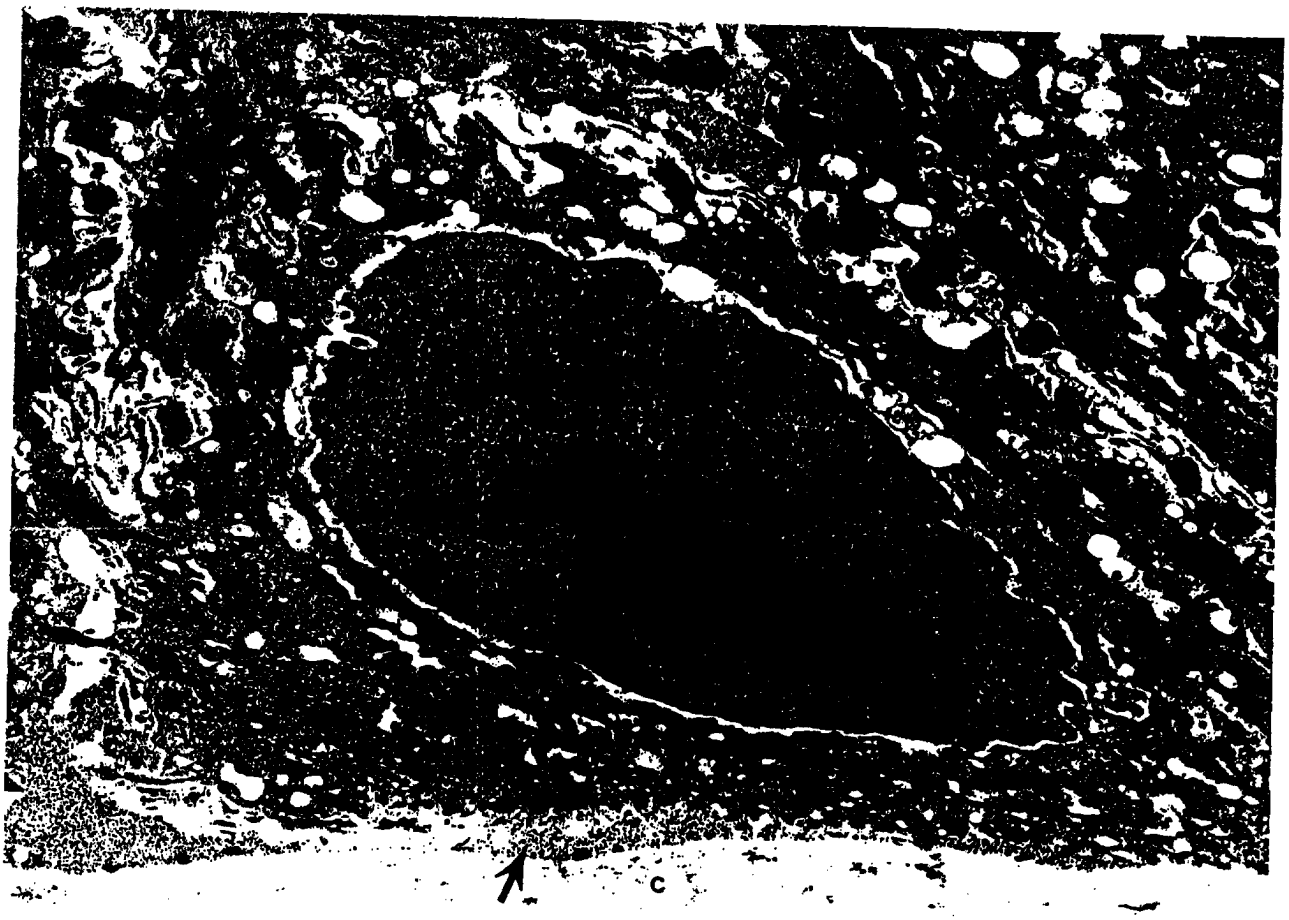

Figure 29. Transmission electron micrograph showing the amorphous ground substance (arrow) between the basement membrane and dermal collagen (c). MT, Day 3, 13,200X

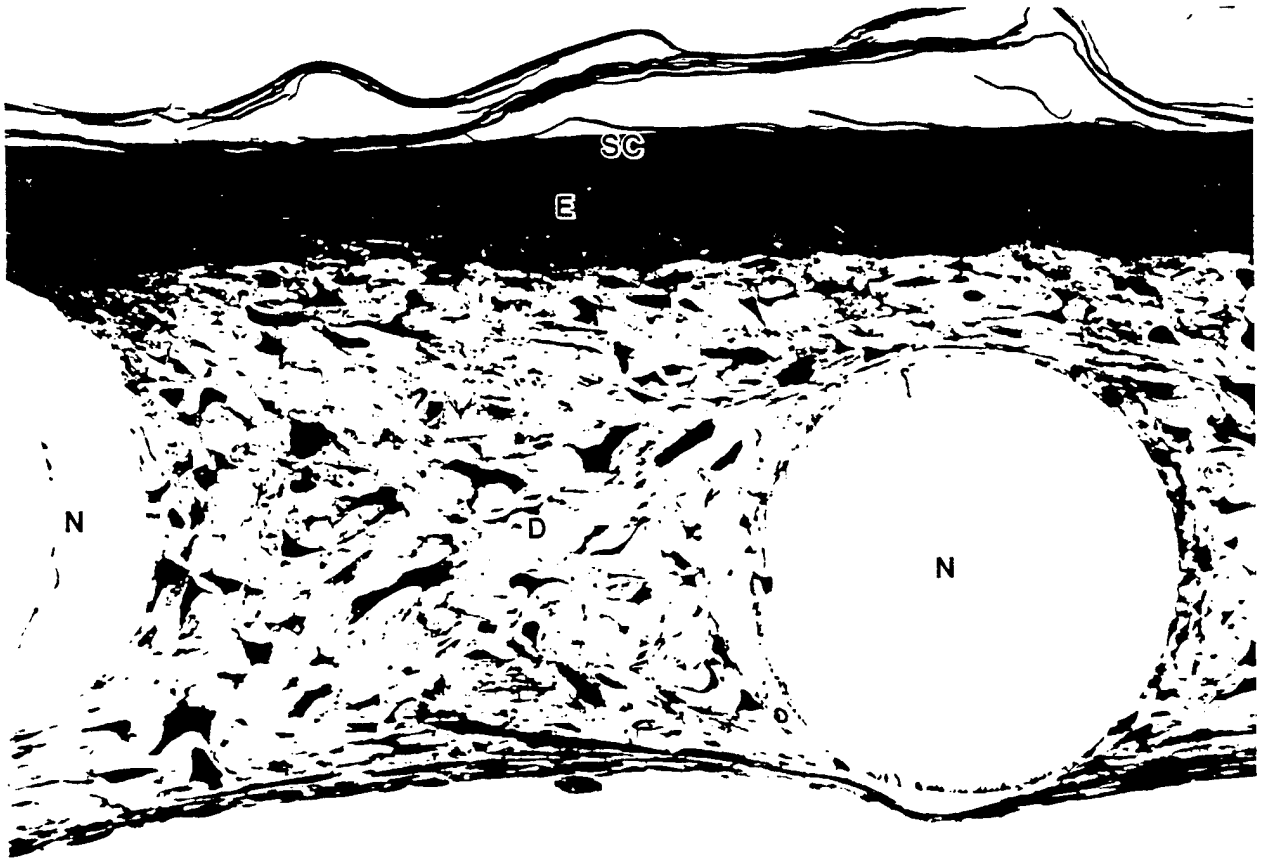

Figure 30. Light micrograph showing a compact stratum corneum (SC), a well developed epidermis (E), and a thick dermis (D). Note cross-section of nylon fibers (N). ATS, Day 0, 400X

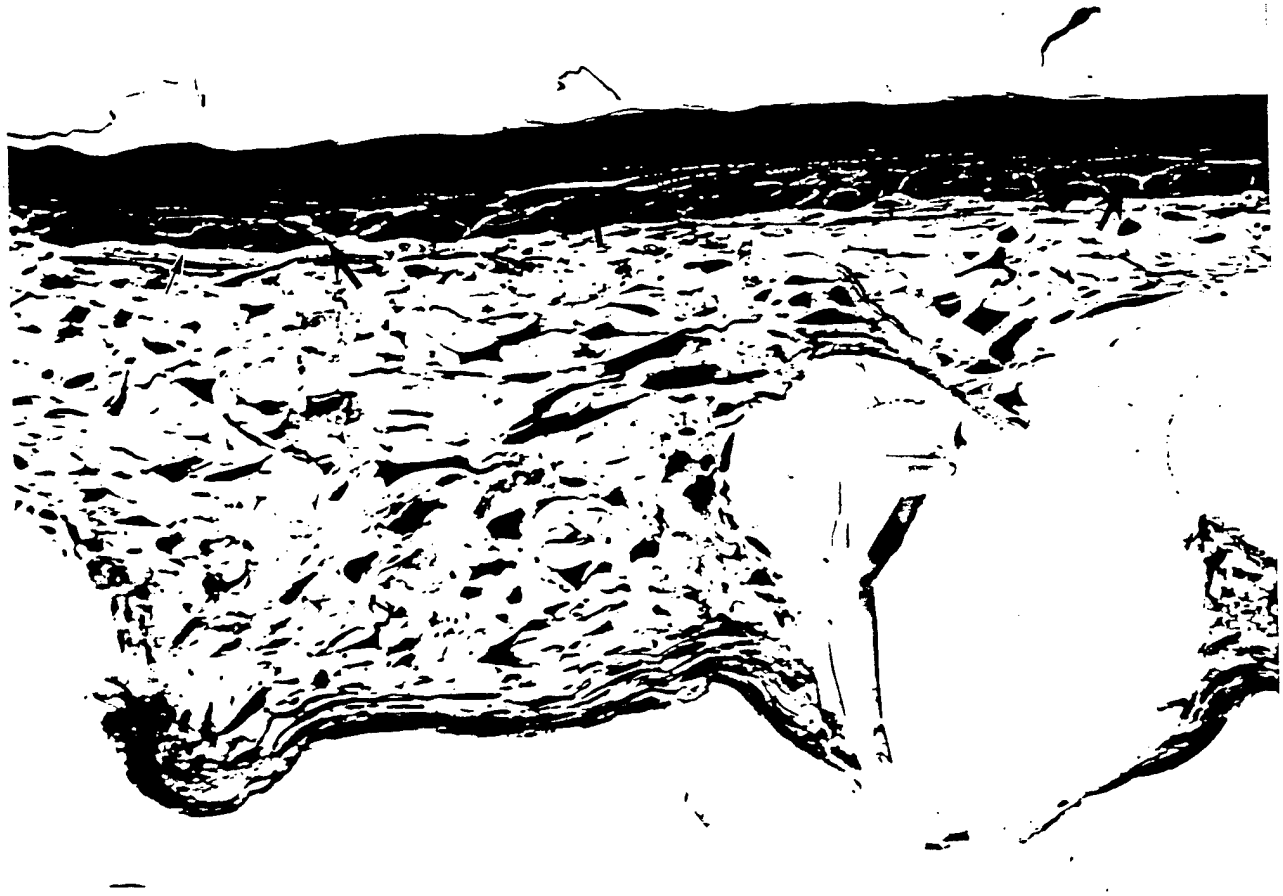

Figure 31. Light micrograph exhibiting separation of the basement membrane (small arrow). Note intercellular epidermal edema (arrows). ATS, Day 0, 400X

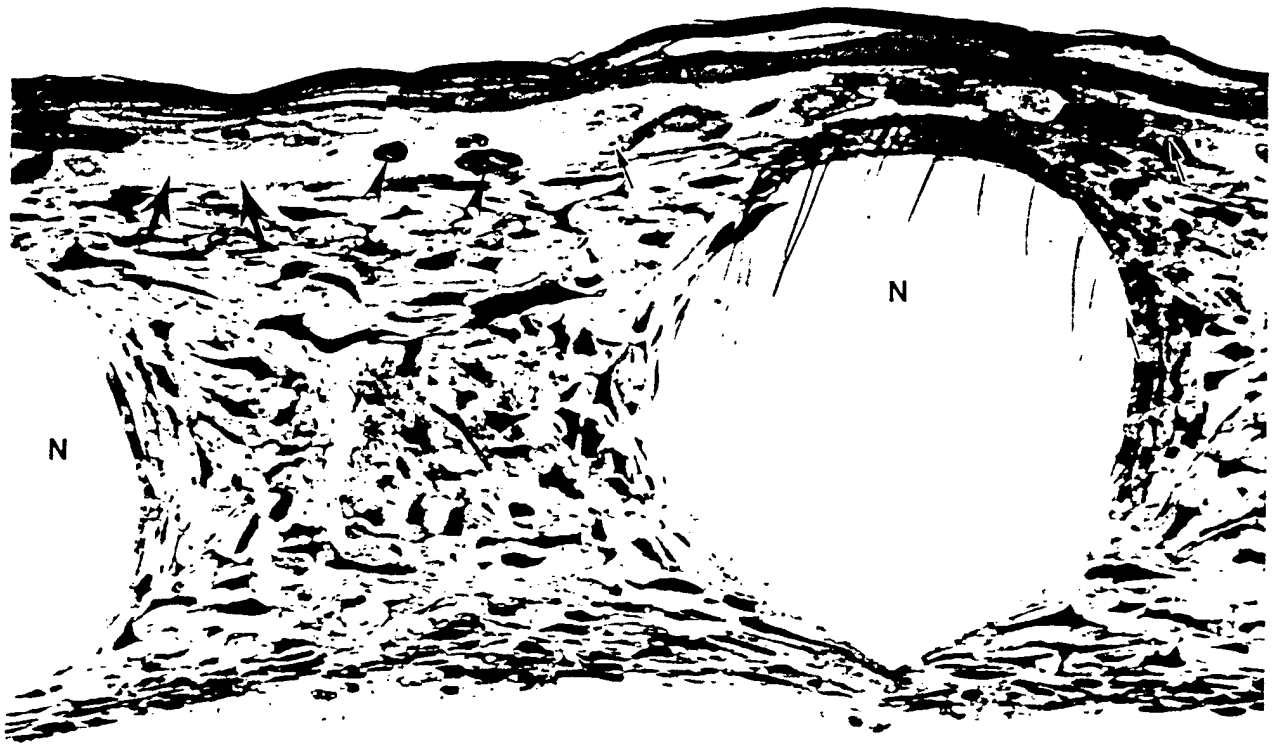

Figure 32. Light micrograph showing dark basal cells (arrowheads), vacuoles (small arrows), and epidermal-dermal separation (large arrows). Note compression of fibroblasts surrounding the nylon fiber (N). ATS, Day 2, 300X

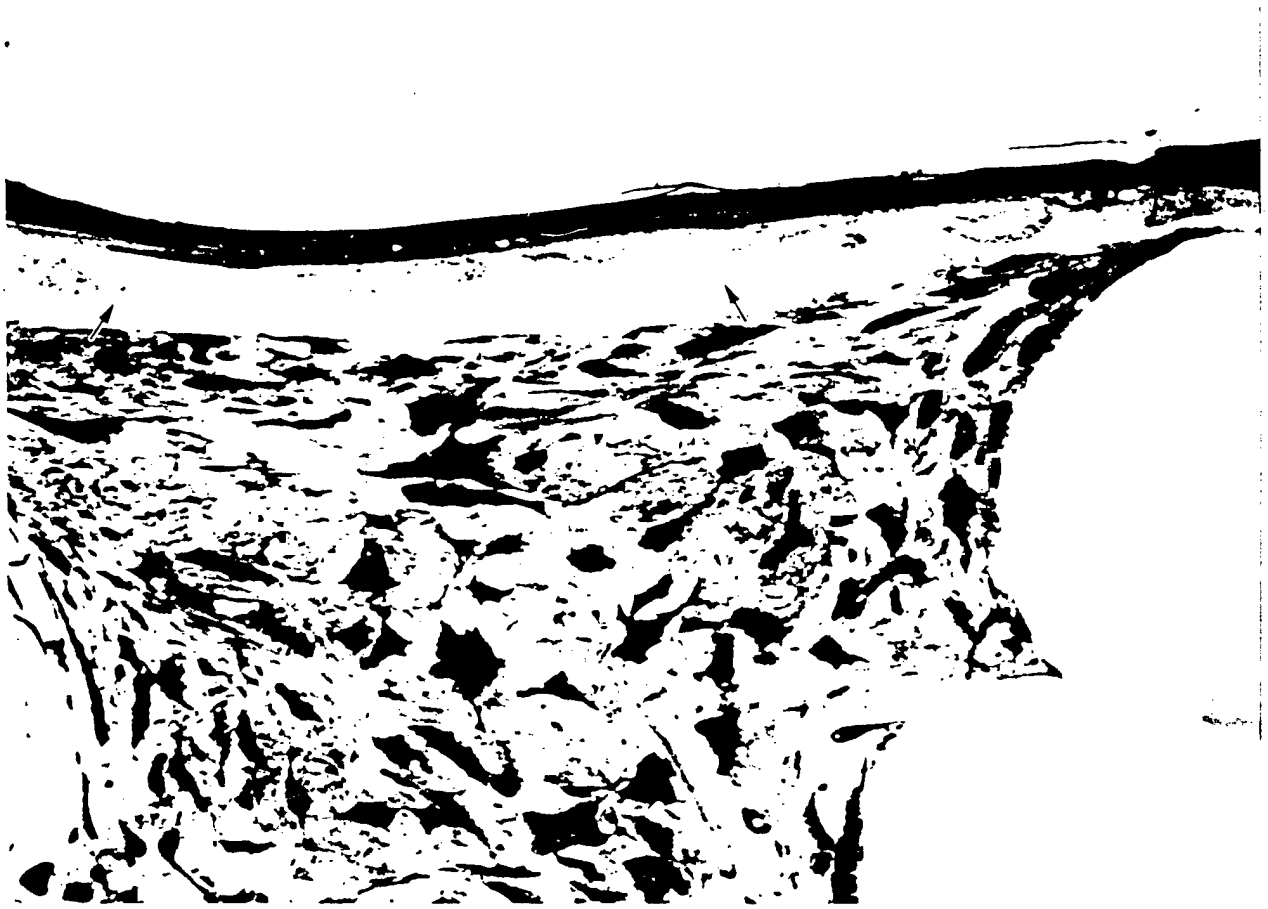

Figure 33. Light micrograph showing epidermal necrosis (arrows). ATS, Day 3, 630X

Figure 34. Transmission electron micrograph of microvillus processes (arrows) extending from the outermost layers of the stratum corneum (SC). ATS, Day 0, 108,500X

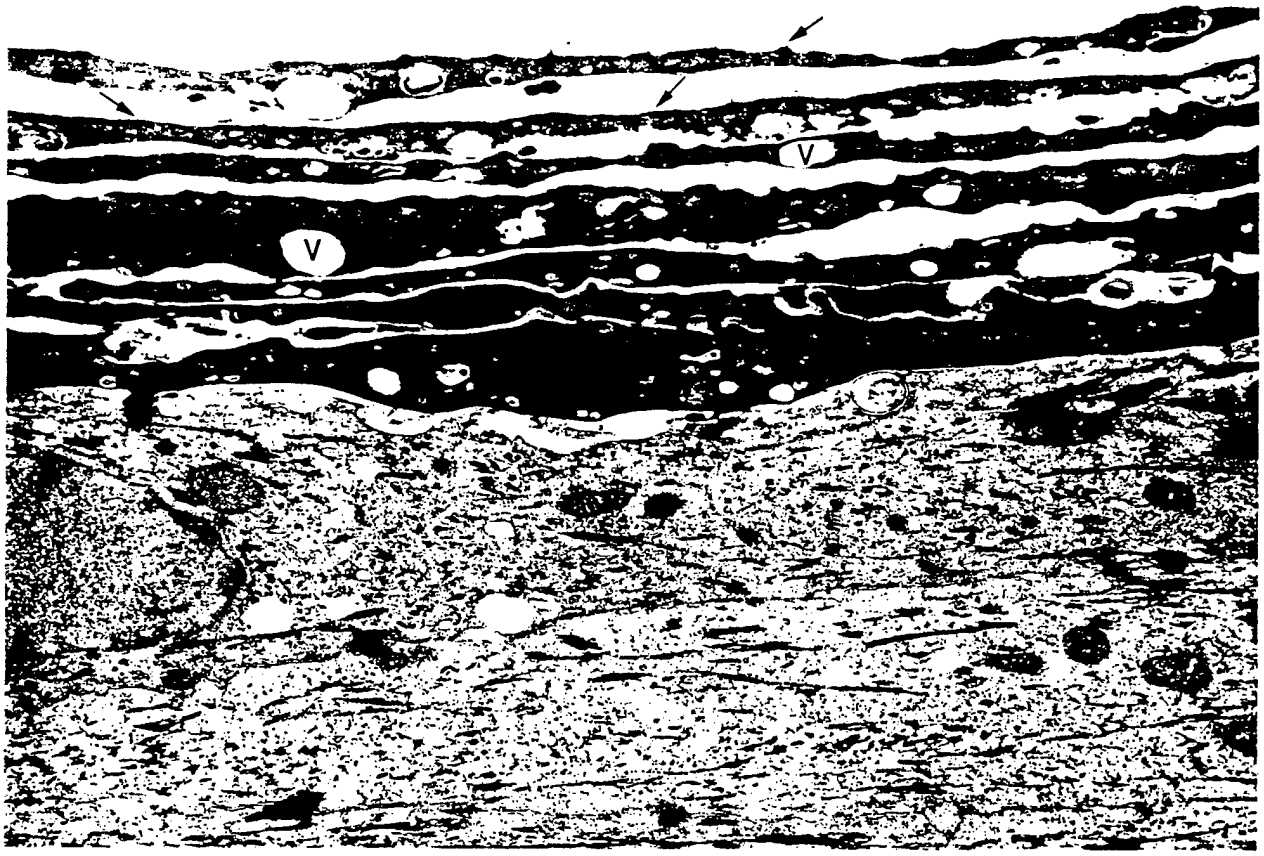

Figure 35. Transmission electron micrograph depicting vacuoles (V) in the stratum corneum cell layers. Note microvillus processes (arrows) on the outermost layers of the stratum corneum. ATS, Day 0, 17,040X

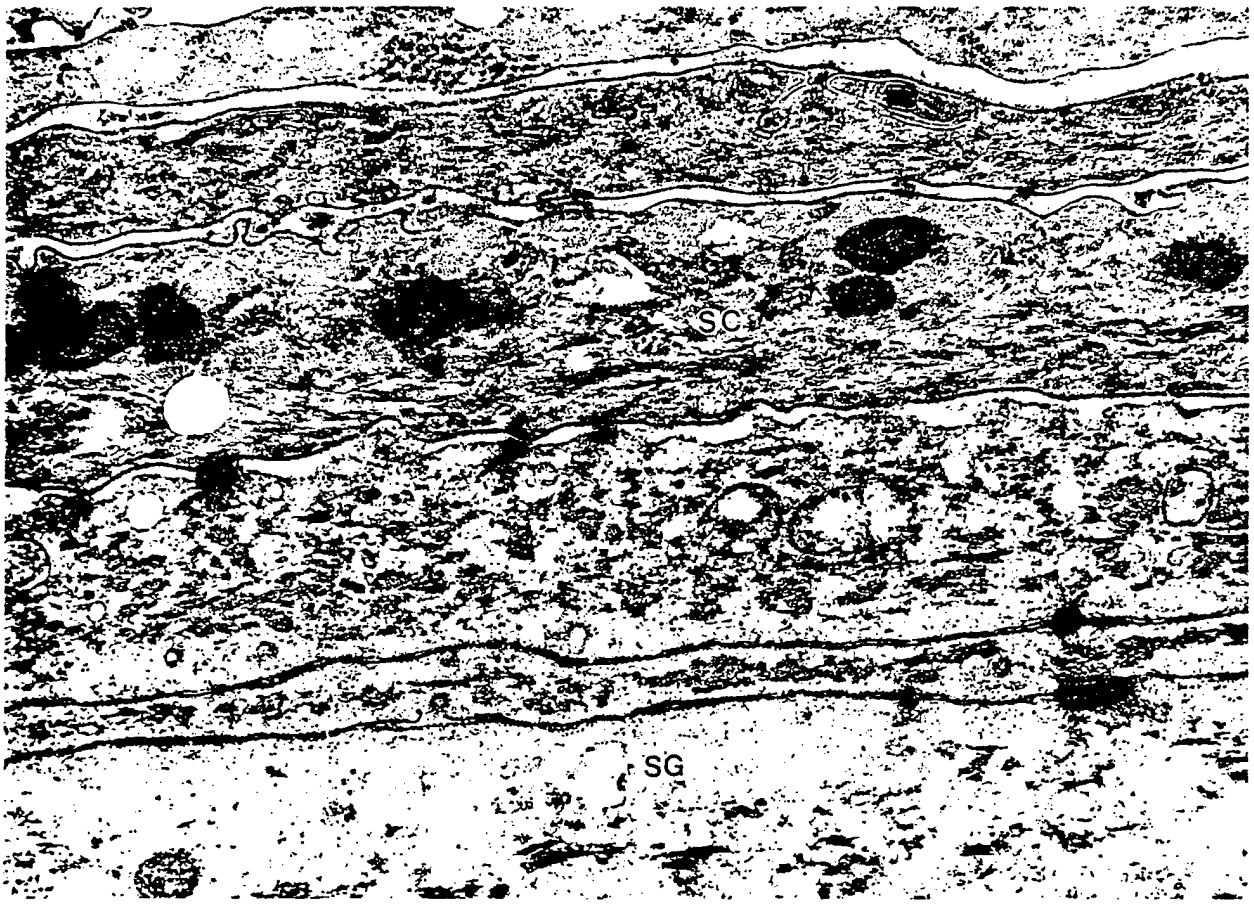

Figure 36. Transmission electron micrograph of a poorly differentiated stratum corneum (SC) and stratum granulosum (SG). ATS, Day 0, 31,200X

Figure 37. Transmission electron micrograph of the stratum spinosum (SS) cell layers containing desmosomes (d), mitochondria (M), and focal areas devoid of cytoplasm (*). ATS, Day 0, 18,460X

Figure 38. Transmission electron micrograph showing a poorly developed stratum spinosum (SS). ATS, Day 0, 11,000X

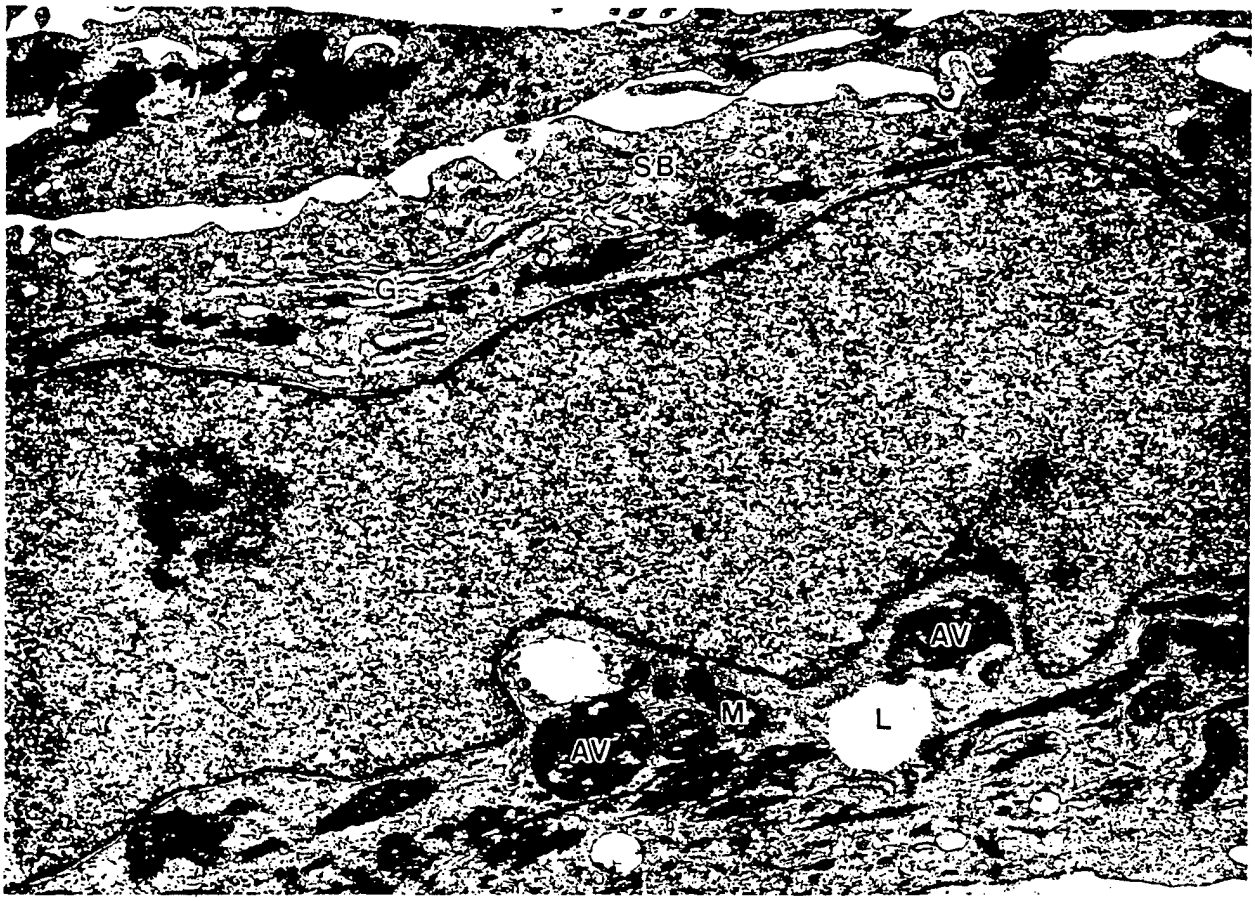

Figure 39. Transmission electron micrograph of a stratum basale (SB) cell containing mitochondria (M), Golgi (G), lipid droplets (L), and autophagic vacuoles (AV). ATS, Day 0, 17,750X

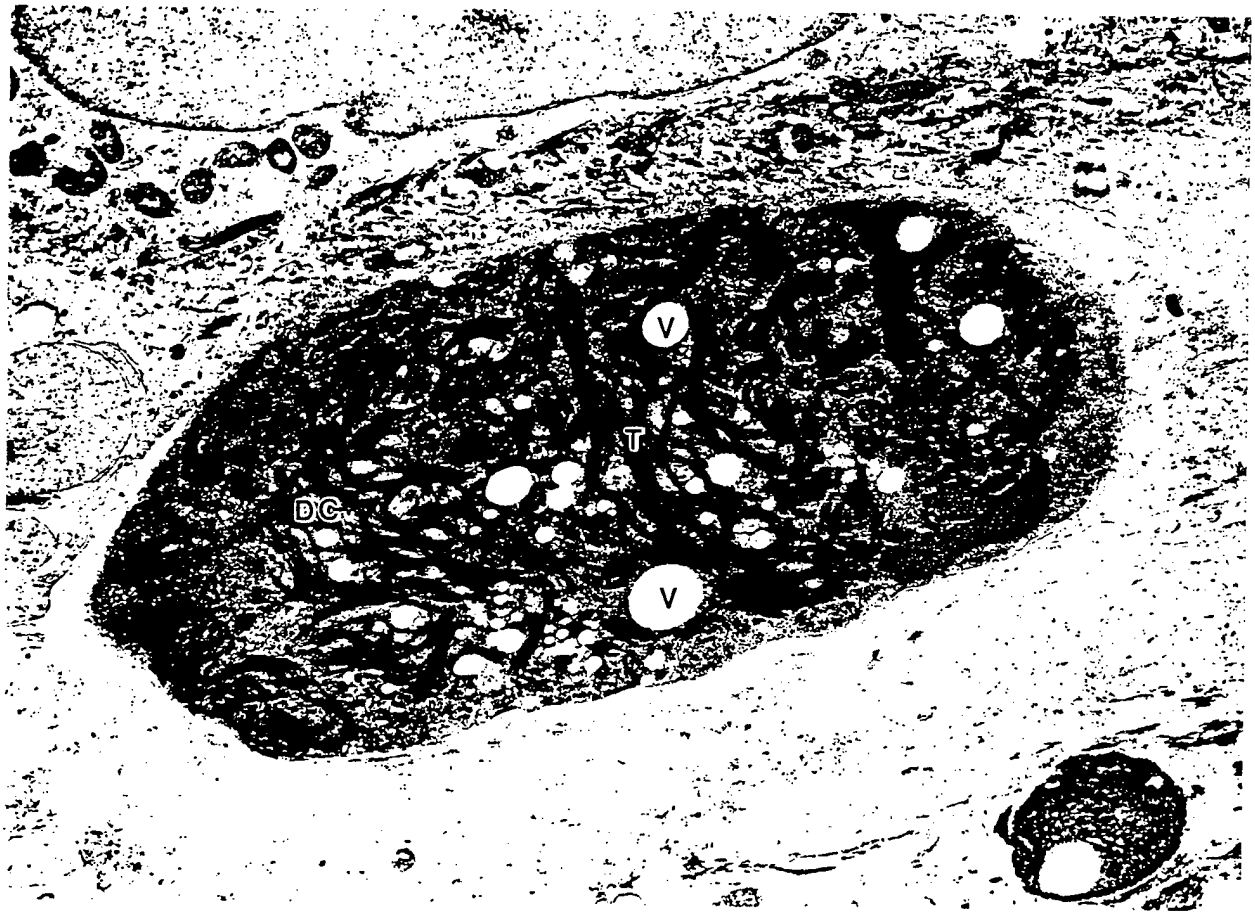

Figure 40. Transmission electron micrograph of a dyskeratotic cell (DC) within the stratum basale layer. Note vacuoles (V) and tonofilaments (T). ATS, Day 0, 13,200X

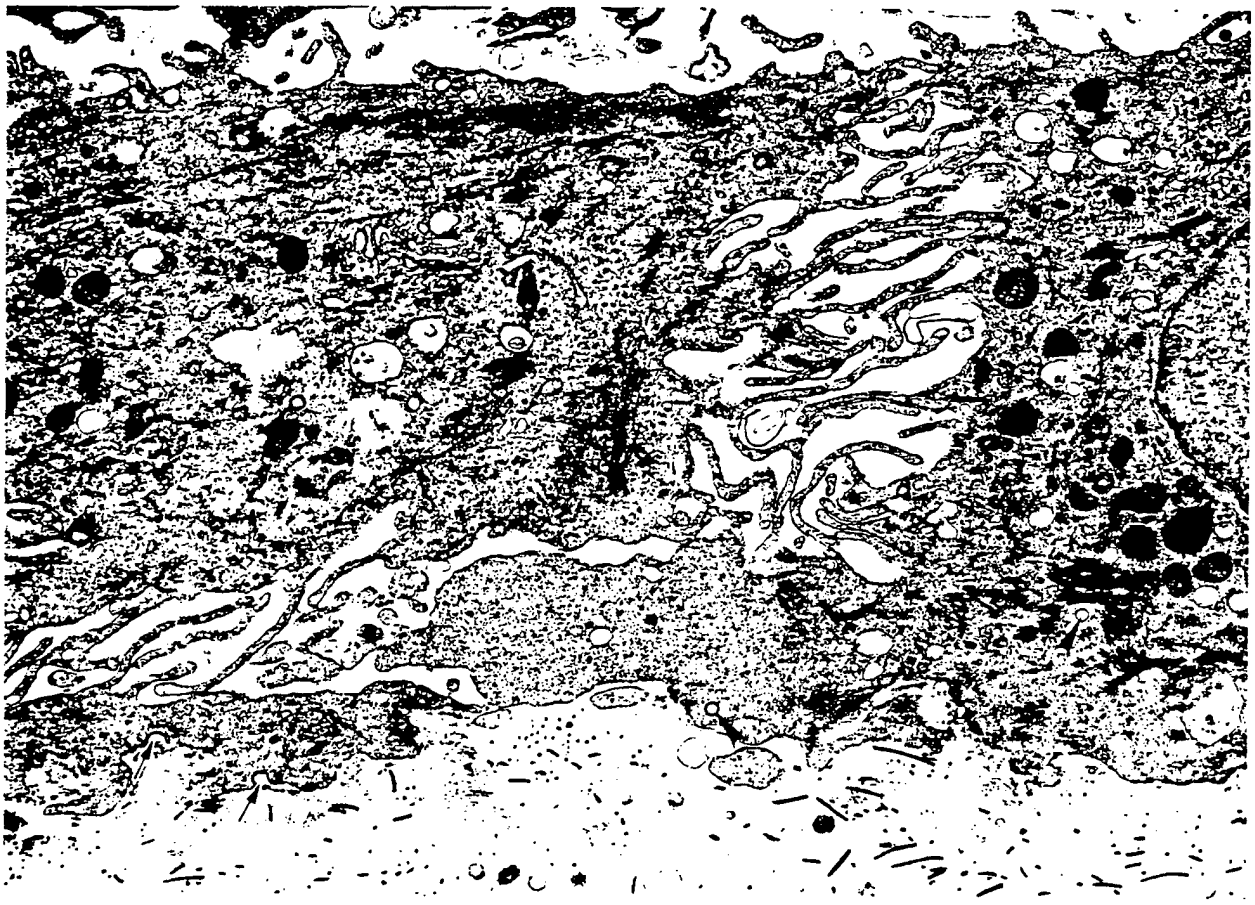

Figure 41. Transmission electron micrograph of endocytotic invaginations (arrows) of the plasma membrane and vesicles within the cytoplasm (arrowheads). ATS, Day 0, 13,200X

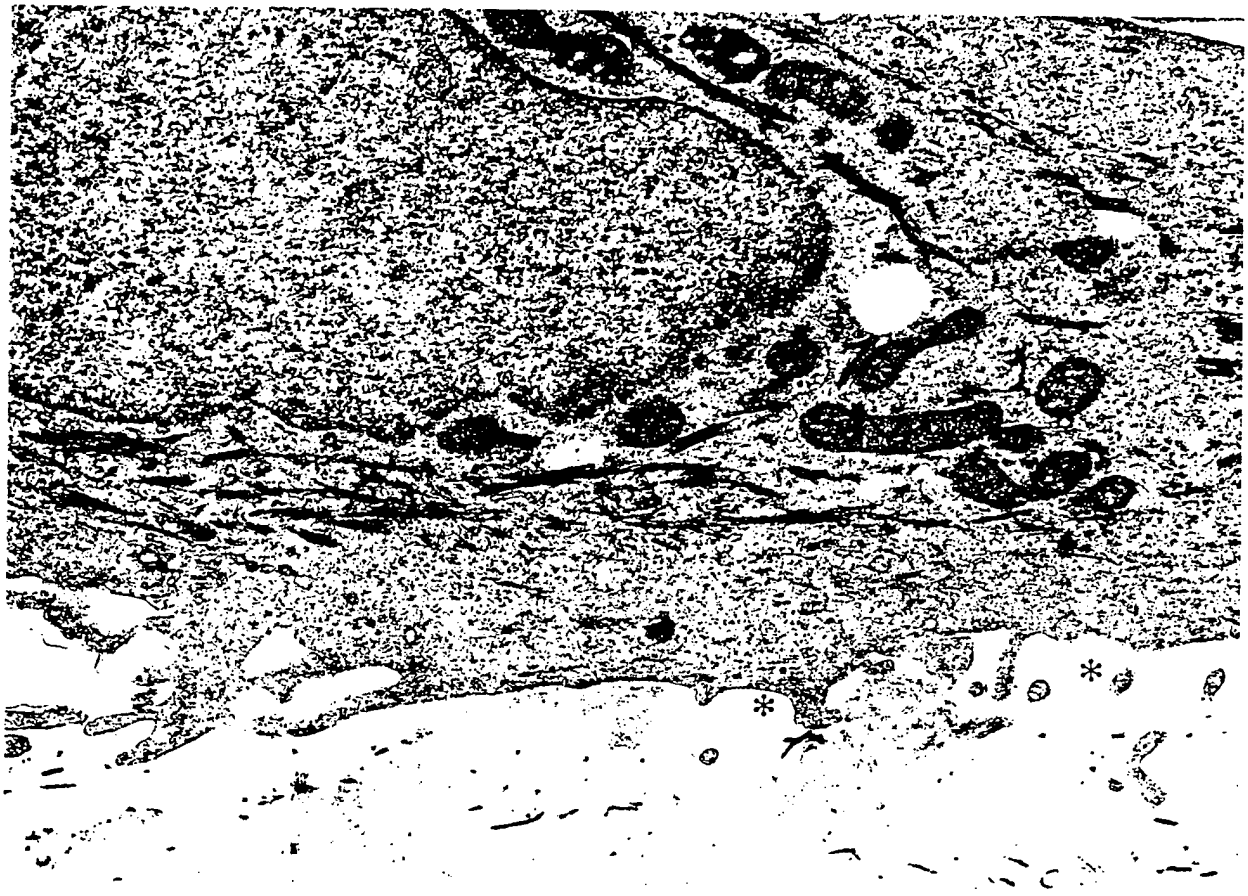

Figure 42. Transmission electron micrograph showing areas devoid of basement membrane. Note spaces (*) immediately beneath the basal cell. ATS, Day 0, 19,880X

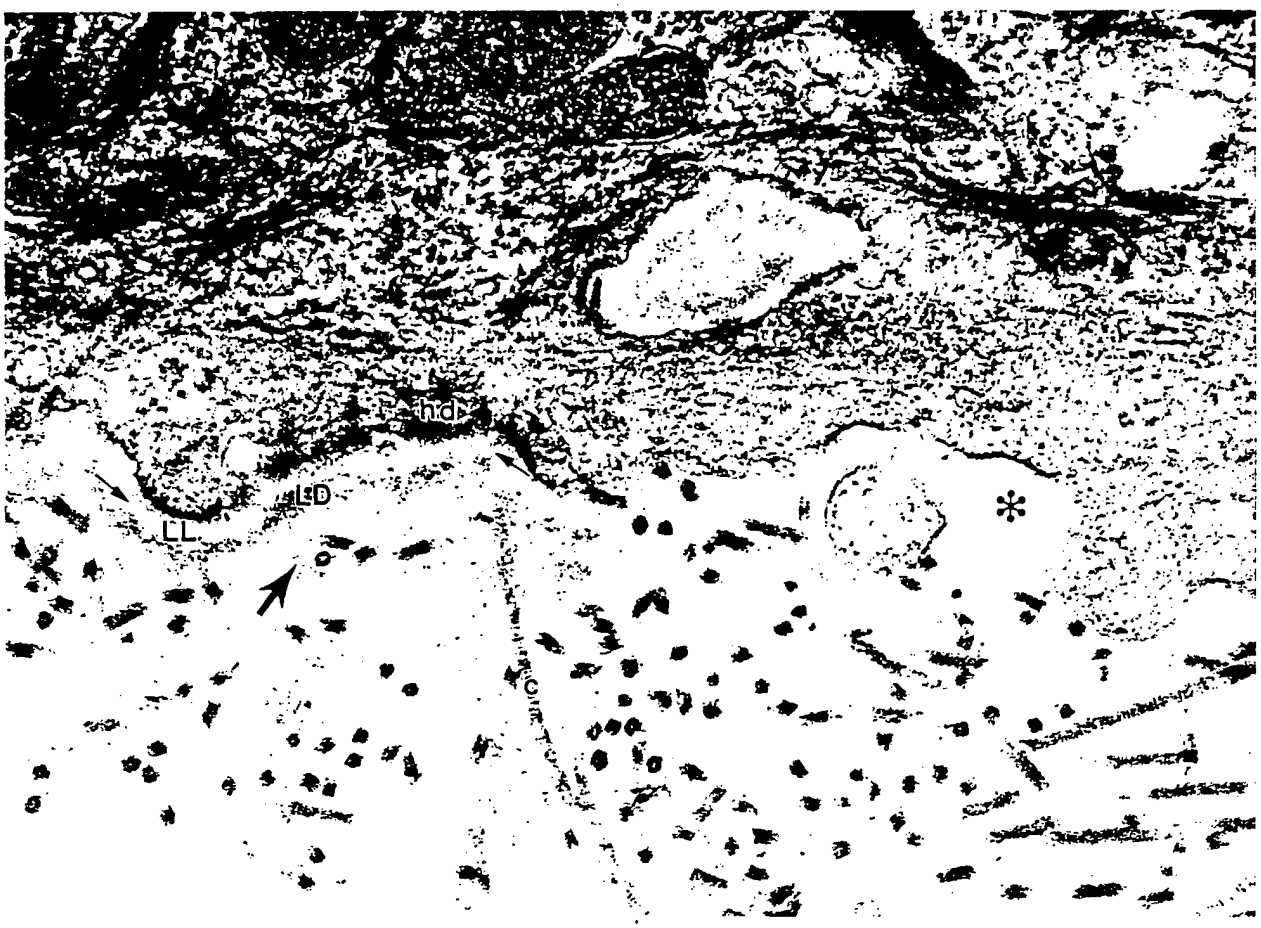

Figure 43. Transmission electron micrograph depicting an empty space (*), hemidesmosomes (hd), lamina lucida (LL), lamina densa (LD), anchoring filaments (small arrows), anchoring fibrils (large arrows), and dermal collagen (c). ATS, Day 0, 54,600X

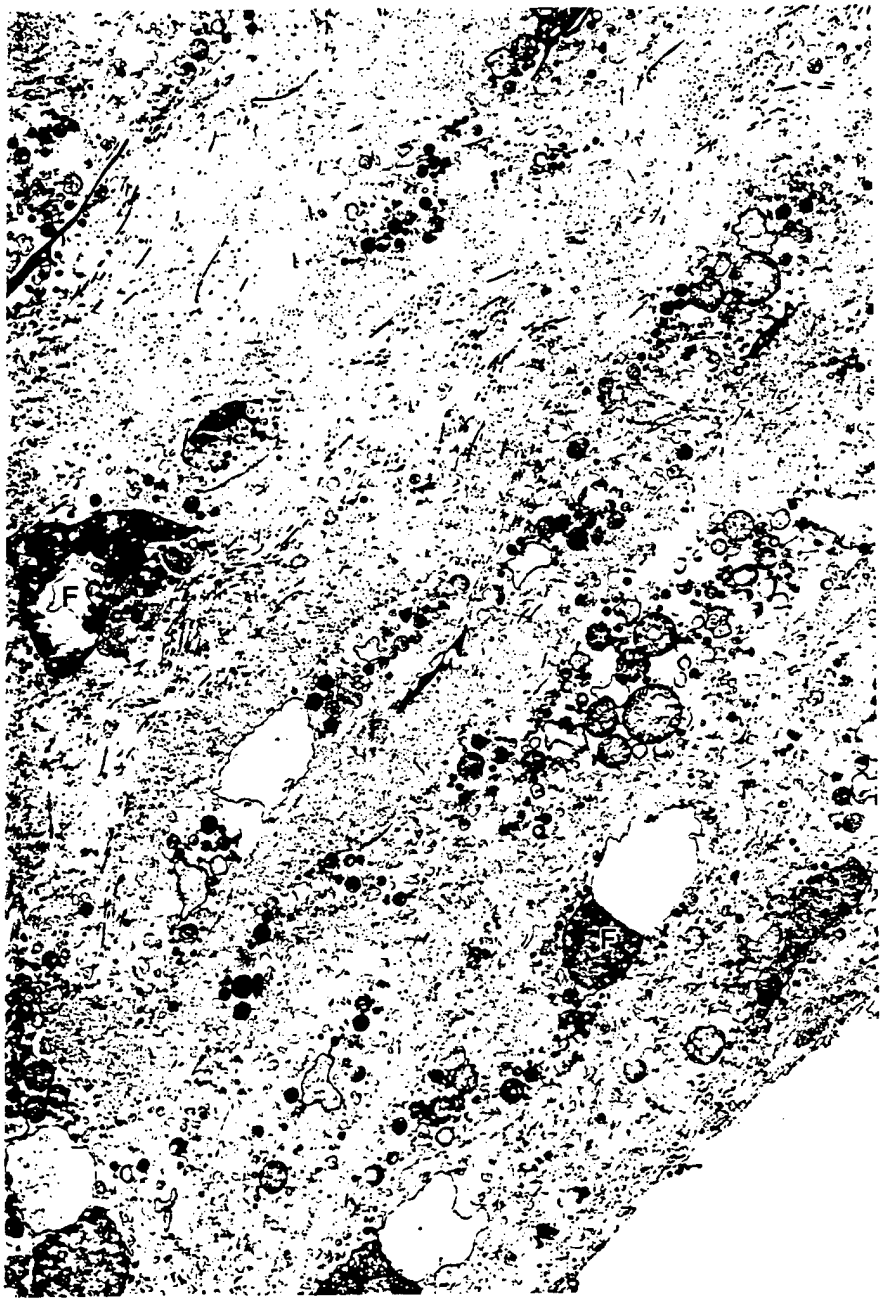

Figure 44. Transmission electron micrograph showing necrotic fibroblasts (F) in the seed layer. ATS, Day 0, 4,980X

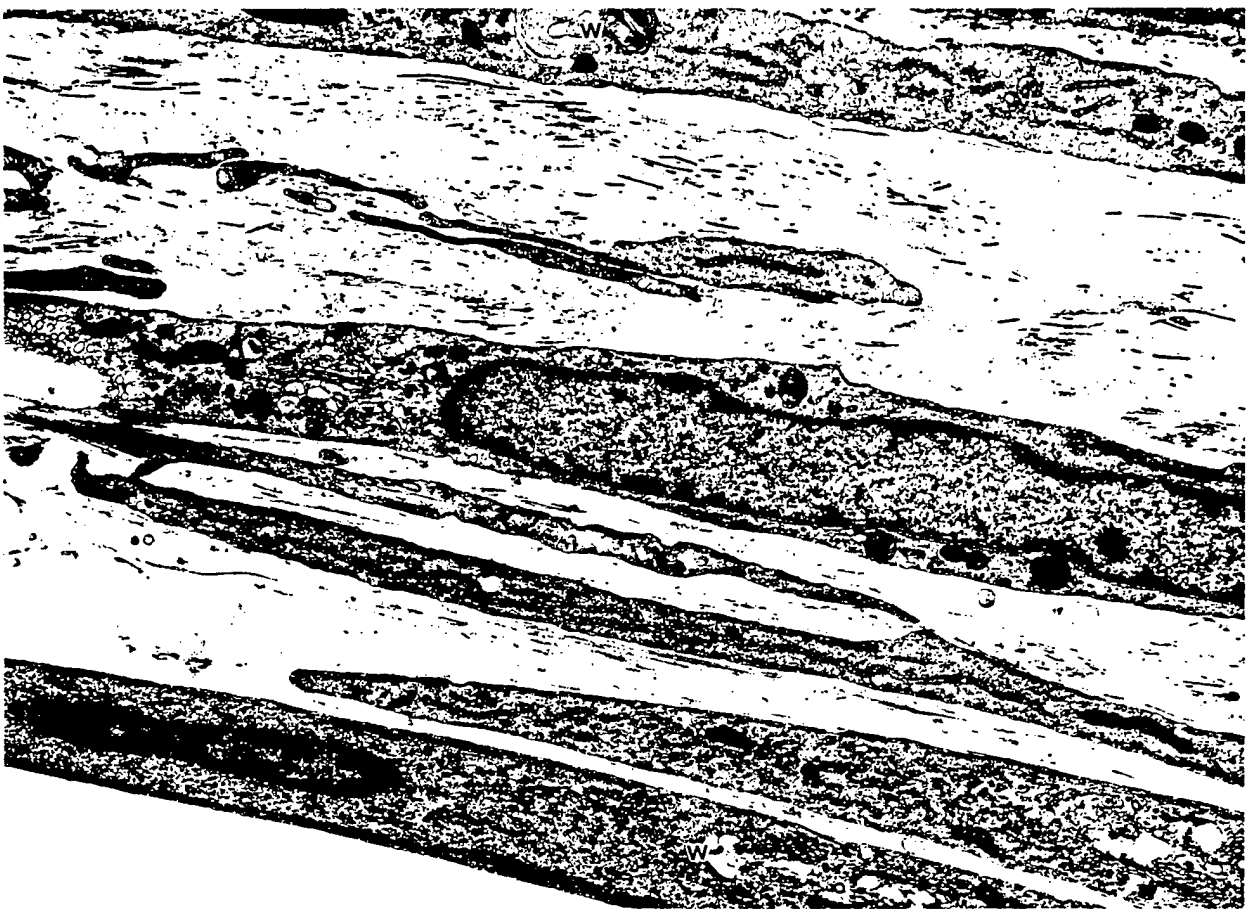

Figure 45. Transmission electron micrograph of the seed layer with fibroblasts containing whorls (w) and typical organelles. ATS, Day 0, 11,440X

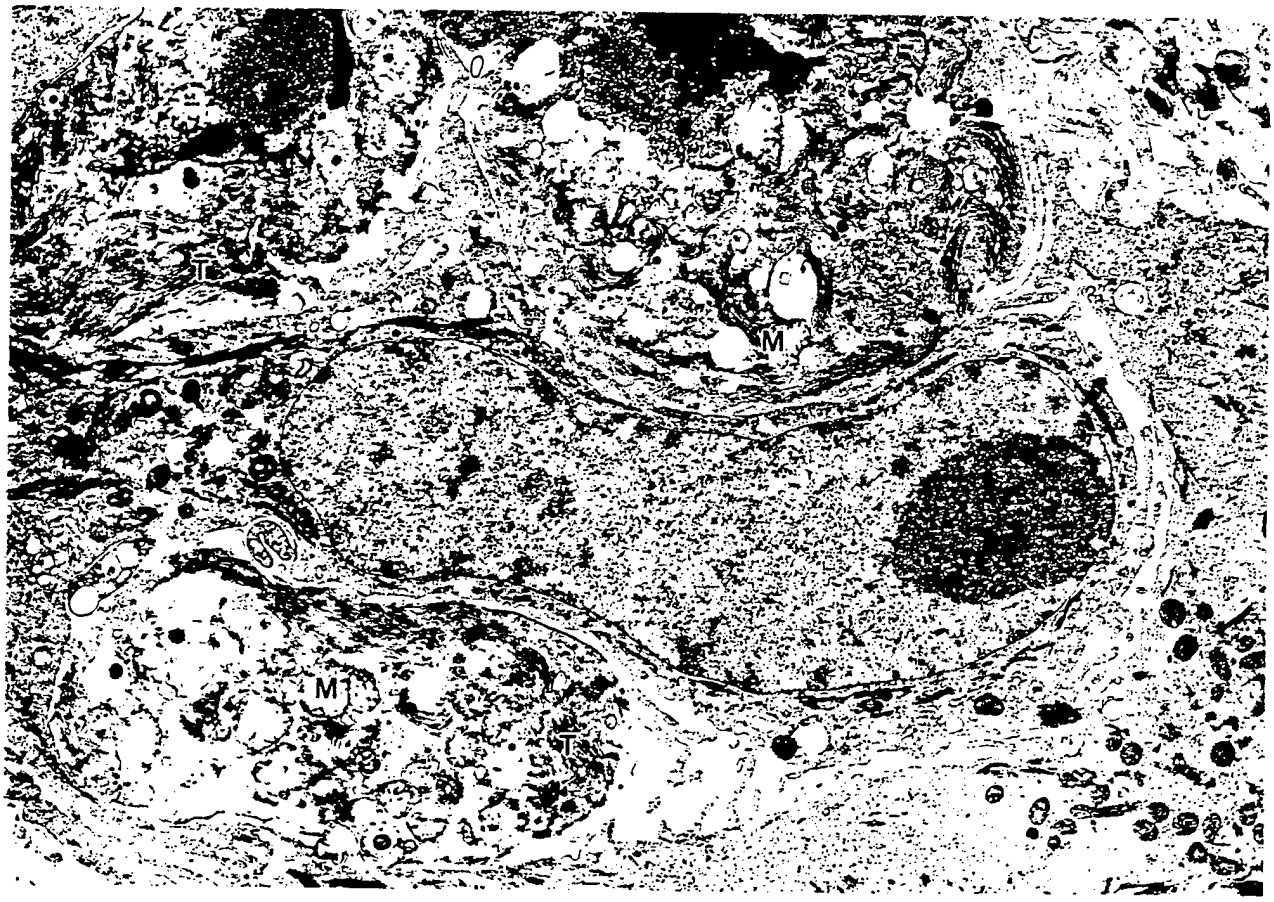

Figure 46. Transmission electron micrograph of several necrotic cells containing tonofilaments (T) and damaged mitochondria (M). ATS, Day 1, 11,000X

Figure 47. Transmission electron micrograph of necrotic (nc) and dark basal (bc) cells. ATS, Day 2, 11,000X

Figure 48. Transmission electron micrograph showing an area devoid of basement membrane (large arrows). ATS, Day 2, 52,500X

Figure 49. Transmission electron micrograph showing undifferentiated stratum corneum (SC) and epidermal vacuoles (V). ATS, Day 3, 6,000X

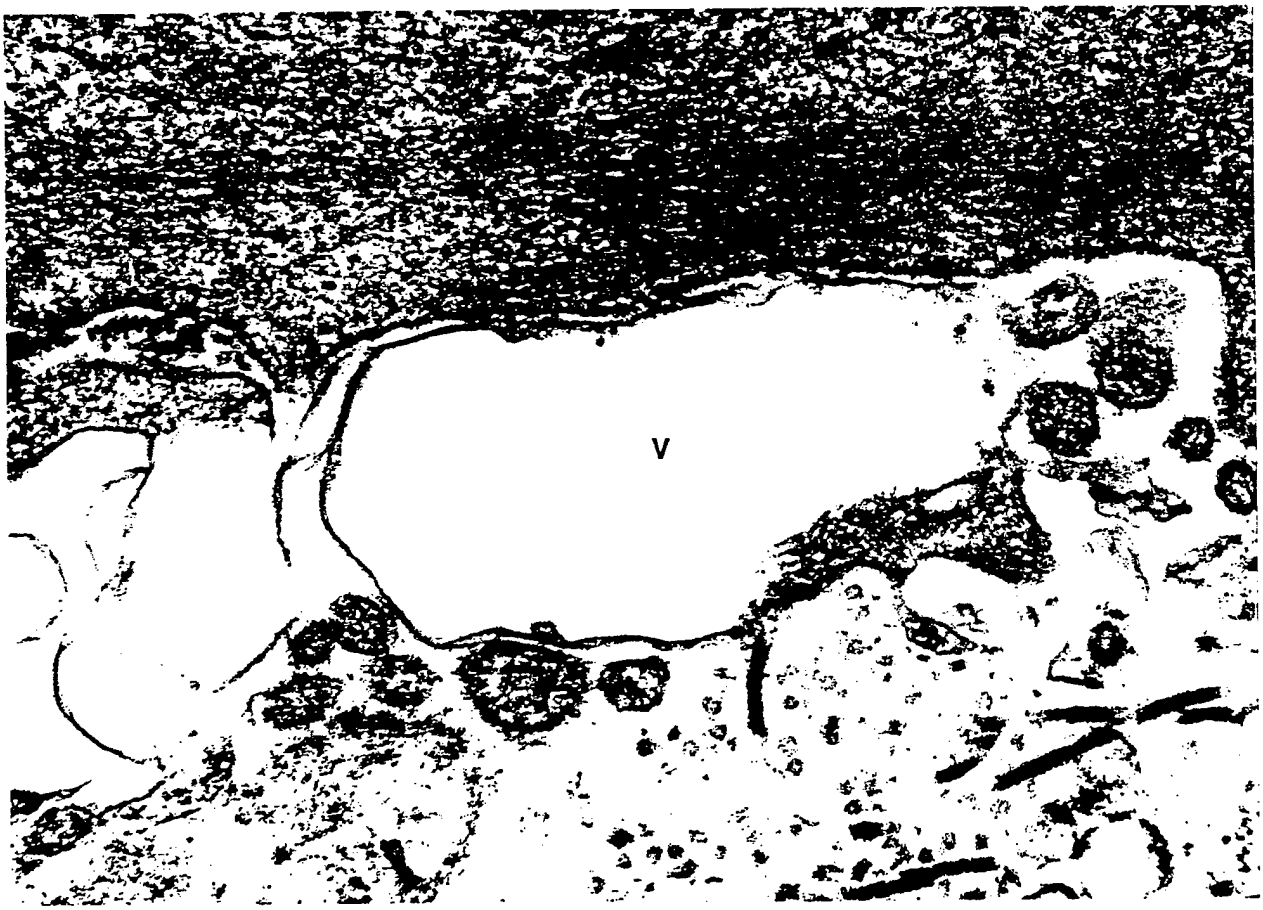

Figure 50. Transmission electron micrograph of a vacuole (V) in the area of the lamina lucida and lamina densa. ATS, Day 3, 54,600X

APPENDIX C

Light Microscopy of Samples of Natural Human Skin Obtained from the Ohio Valley Tissue and Skin Center

THE OHIO STATE UNIVERSITY
UNIVERSITY MEDICAL CENTER

University Hospitals
Department of Internal Medicine
Dermatology

4731 University Hospitals Clinic
456 West 10th Avenue
Columbus, OH 43210-1282

Phone: (614) 293-8111 Administrative
(614) 293-8163 Patient Appointments

November 14, 1994

Thomas H. Snider
Research Scientist Battelle
505 King Avenue
Columbus, OH 43201-2693

Dear Mr. Snider:

I have reviewed MREF Task 91-24 phase I histology slides labeled 2176 Day 0 and 2176 Day 1. The epidermis in day 0 shows more compact keratosis than does the specimen in day 1. Day 1 specimen shows basket weave orthokeratosis which is characteristic of human skin as opposed to compact keratosis. I cannot explain this finding. The squamous cells in day 0 and day 1 specimens are indistinguishable. The spaces surrounding the blood vessels in day 1 specimen are greater than those in day 0 specimen. Sweat gland epithelium in day 1 specimen showed degenerative changes when compared with day 0 specimens. In sum, the difference between day 0 and day 1 specimens are noted in the dermis with widening of perivascular spaces and degeneration of sweat apparatus epithelium.

Sincerely,

Arthur E. Pellegrini, M.D.
Professor of Dermatology and Clinical Pathology
Director (Acting), Division of Dermatology

pellegrini.snider

APPENDIX D

Tables

Table D-1. Descriptive Statistics on the Thickness (μm) of Tissue Layers in Advanced Tissue Sciences™ Human Skin Equivalent Model ZK1300

			Stratum Corneum	Epidermis	Total Skin
Lot No.	Day	N	Mean	Mean	Mean
8041-3-104-11-13B	0	2	6	41	151
8043-3-104-11-15D	0	4	10	42	162
	1	4	8	36	151
	2	4	10	34	154
8045-3-138-11-14E	0	4	11	51	179
8046-3-138-11-14B	Before Shipment	2	7	53	167
	0	4	7	47	172
Coefficient of Variation	0	4	0.27	0.10	0.07

Table 2. Descriptive Statistics on Viability Indices for Advanced Tissue Sciences™ Human Skin Equivalent Model ZK1300

		Glucose Utilization (g/L/hr)		Lactate Production (g/L/hr)		Mean Lactate/ Glucose	MTT Assay Results (Optical Density$_{540}$)		
Lot No.	Day	N	Mean Slope	N	Mean Slope	Ratio	N	Mean	Std
8041-3-104-11-13B	0	4	0.08	4	0.09	1.11	4	1.15	0.05
	1	4	0.14	4	0.09	0.64	4	0.95	0.03
	2	4	0.21	4	0.09	0.45	4	0.98	0.02
8043-3-104-11-15D	0	3	0.24	3	0.08	0.32	3	0.76	0.05
	1	3	0.26	3	0.10	0.39	3	0.51	0.12
	2	3	0.26	3	0.08	0.33	3	0.81	0.04
8045-3-138-11-14E	0	3	0.21	3	0.04	0.21	3	0.85	0.07
	1	3	0.24	3	0.08	0.33	3	0.94	0.05
	2	3	0.24	3	0.10	0.41	3	1.01	0.12
8046-3-138-11-14B	0	3	0.24	3	0.06	0.27	3	0.77	0.02
	1	3	0.23	3	0.09	0.37	3	0.94	0.04
	2	3	0.26	3	0.08	0.33	3	1.09	0.07
Coefficient of Variation	0		0.39		0.28	0.90		0.21	
	1		0.24		0.10	0.32		0.26	
	2		0.10		0.07	0.16		0.12	

APPENDIX E

Light Microscopy of Samples of Advanced Tissue Sciences Human Skin Equivalent Model ZK1300

MORPHOLOGICAL ANALYSIS OF ATS LIVING SKIN EQUIVALENTS

Hematoxylin and eosin stain paraffin sections of Advanced Tissue Sciences samples were analyzed and reported below. The thickness of the stratum corneum, epidermis, and total skin was measured at three random sites and summarized in Table 1.

Lot 13B8041-3-104-11/ Day 0
Slides 1A and 1B: The stratum corneum (SC) was very compact. However, the stratum basale (SB) cells in 1A were flattened, while those in 1B were large and well differentiated. Intercellular edema was present between the SB and stratum spinosum (SS) cell layers. While the SC consistently measured 6μm in thickness, the epidermis (28-56μm) and full skin thickness (125-178μm) was variable.

Lot 8043-3-104-11-15D/ Day 0
Slides 5A, 6A, 7B, and 8A: The SC in these samples were poorly developed. The SB layer was mostly necrotic in all of these samples. Sample 6A had epidermis growing down into the dermis and surrounding the nylon mesh fibers. The poorly developed SC varied in thickness from 3-19μm. The epidermal and total skin thickness was fairly consistent, with the exception of a few outliers.

Lot 8043-3-104-11-15D/ Day 1
Slides 9B,10B, 11B and 12B: The SC was poorly developed. Samples 9B and 12B contained intercellular edema between SB and SS layers, while samples 10B and 11B had intracellular edema within the SB and SS cells. Focal necrotic and dyskeratotic SB cells were present in most samples. Focal concentric layers of dyskeratotic cells were found in the epidermis and dermis of 10B. In sample 12B, epidermal cells were found growing down into the dermis and surrounding the nylon mesh fibers. The poorly developed SC varied in thickness from 3-22μm. While epidermal thickness was highly variable within several samples, overall skin thickness was fairly consistent.

Lot 8043-3-104-11-15D/ Day 2
Slides 13A, 14A, 15B, and 16B: The SC was poorly developed. Samples 13A and 14A primarily consisted of necrotic epidermal cells. A few dyskeratotic SB cells and epidermal-dermal separation were found in sample 16B. A band of dermal fibroblasts weaves a zig zag pattern around nylon mesh fibers in sample 14A. Samples 13A and 14A had a much thicker SC than 15B and 16B. Epidermal and overall thickness measurements were fairly consistent within and between samples.

Lot 8043-3-138-11-14E/ Day 0
Slides 17B, 18A, 19B, and 20B: The SC was well developed and compact. All samples displayed intercellular edema between the SB and SS cells, but 17B and 18A showed the best morphological characteristics. Samples 18A and 20B contained concentric epidermal cells in the dermis that possibly represents an early stage of hair follicle development. Sample 19B showed epidermal cells growing down into the dermis surrounding the nylon

mesh fibers. Little SC, epidermal, and overall skin thickness variability was found within and between samples.

Lot 8046-3-138-11-14B/ Day 0

Slides 29B, 30A, 31A, and 32B: The SC was compact. All samples contained intercellular edema between the SB and SS cells, as well as focal areas of necrosis and dyskeratosis within the SB cells. Samples 29B and 31A had focal areas of concentric epidermal cells within the dermis. Sample 31A showed epidermal cells growing down and around the nylon mesh fibers. Although the SC thickness varied between 3-11µm, overall thickness was fairly consistent. With few exceptions, the epidermal and overall skin thickness was fairly consistent.

Control A and B: Both samples had a compact SC and very slight intercellular edema in the SB and SS cell layers. Control A had a few dyskeratotic cells in the SB. The SC, epidermal, and total skin thickness was consistent.

In conclusion, the morphology of samples from two different lots at identical time points was variable and the overall morphology very poor. Several Day 1 samples had focal areas that lacked SC. The Day 2 samples primarily consisted of necrotic cells in all epidermal layers and a poorly developed SC layer. Only samples 17B, 18A, control A, and control B appeared fairly normal. Tissue damage and intercellular and intracellular epidermal edema were often so severe that the samples could not be favorably compared to normal skin. Another problem with this *in vitro* skin model is the morphological and thickness inconsistencies noted within and between tissue lots. The mean thickness varied for the SC (6.00 to 11.00µm), the epidermis (41.33 to 50.75), and the total skin (151.00 to 179.08) in the Day 0 samples. The Day 2 SC and total skin thickness was greater than the Day 1, with both falling within the thickness range for the Day 0 measurements. While the Day 1 epidermal thickness was slightly greater than the Day 2, both values fell below the Day 0 range.

The degradative changes within samples of the same time points would make it extremely difficult to accurately assess cutaneous toxicity in this skin model. One would always question whether the observed alterations are inherent in the model or due to cellular toxicity.

TABLE 1 - ATS thickness measurements.

	STRATUM CORNEUM (microns)			SC	SC	EPIDERMIS (microns)			EPIDER	EPIDER	TOTAL SKIN (microns)			TOTL	TOTAL
SAMPLE #	SITE 1	SITE 2	SITE 3	MEAN	STDEV	SITE 1	SITE 2	SITE 3	MEAN	STDEV	SITE 1	SITE 2	SITE 3	MEAN	STDEV
95LM-1A	6	6	6	6.00	0.00	28	33	31	30.67	2.52	133	125	142	133.33	8.50
95LM-1B	6	6	6	6.00	0.00	53	56	47	52.00	4.58	178	170	158	168.67	10.07
MEAN: DAY 0, LOT 13B8041-3-104-11				**6.00**	**0.00**				**41.33**	**3.55**				**151.00**	**9.29**
95LM-5A	6	8	6	6.67	1.15	28	47	47	40.67	10.97	167	167	147	160.33	11.55
95LM-6A	11	11	14	12.00	1.73	47	44	50	47.00	3.00	178	178	175	177.00	1.73
95LM-7B	14	19	11	14.67	4.04	50	56	39	48.33	8.62	170	181	170	173.67	6.35
95LM-8A	8	3	8	6.33	2.89	36	19	36	30.33	9.81	133	125	150	136.00	12.77
MEAN: DAY 0, LOT 8043-3-104-11-15D				**9.92**	**2.45**				**41.58**	**8.10**				**161.55**	**8.10**
95LM-9B	6	11	22	13.00	8.19	31	39	75	48.33	23.44	153	145	170	156.00	12.77
95LM-10B	11	11	6	9.33	2.89	44	50	36	43.33	7.02	167	167	158	164.00	5.20
95LM-11B	6	6	3	5.00	1.73	33	28	14	25.00	9.85	145	125	128	132.67	10.79
95LM-12B	3	3	3	3.00	0.00	33	28	22	27.67	5.51	164	139	150	151.00	12.53
MEAN: DAY 1, LOT 8043-3-104-11-15D				**7.58**	**3.20**				**36.08**	**11.45**				**150.12**	**10.32**
95LM-13A	14	19	17	16.67	2.52	39	36	50	41.67	7.37	150	142	128	140.00	11.14
95LM-14A	14	14	17	15.00	1.73	33	36	44	37.67	5.69	153	142	175	156.67	16.80
95LM-15B	3	6	6	5.00	1.73	22	25	31	26.00	4.58	167	147	161	158.33	10.26
95LM-16B	8	3	3	4.67	2.89	28	33	36	32.33	4.04	167	150	167	161.33	9.81
MEAN: DAY 2, LOT 8043-3-104-11-15D				**10.33**	**2.22**				**34.42**	**5.42**				**154.78**	**12.00**
95LM-17B	8	8	11	9.00	1.73	53	50	53	52.00	1.73	195	181	178	184.67	9.07
95LM-18A	11	11	14	12.00	1.73	56	44	61	53.67	8.74	186	175	178	179.67	5.69
95LM-19B	11	8	11	10.00	1.73	47	44	56	49.00	6.24	178	178	175	177.00	1.73
95LM-20B	11	14	14	13.00	1.73	36	53	56	48.33	10.79	167	175	183	175.00	8.00
MEAN: DAY 0, LOT 8045-3-136-11-14E				**11.00**	**1.73**				**50.75**	**6.87**				**179.78**	**6.12**
95LM-29B	11	11	8	10.00	1.73	50	36	58	48.00	11.14	222	181	209	204.00	20.95
95LM-30A	6	8	8	7.33	1.15	56	44	50	50.00	6.00	167	173	167	170.67	6.35
95LM-31A	8	8	6	7.33	1.15	78	53	36	55.67	21.13	164	167	153	161.33	7.37
95LM-32B	3	6	3	4.00	1.73	33	39	31	34.33	4.16	147	167	147	153.67	11.55
95LM-COA	6	8	8	7.33	1.15	50	44	56	50.00	6.00	178	161	167	168.67	8.62
95LM-COB	6	8	6	6.67	1.15	56	61	50	55.67	5.51	167	170	161	166.00	4.58
MEAN: DAY 0, LOT 8046-3-136-11-14B				**7.11**	**1.35**				**48.94**	**8.99**				**170.72**	**9.90**